Immunomodulation by diet

Individual differences in sensitivity in layer hens

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This research was conducted under the auspices of the Graduate School of the Wageningen Institute of Animal Science (WIAS).

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Thesis
submitted in partial fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Doctorate Board
to be defended in public
on Friday 20 November 2009
at 4 PM in the Aula.

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PhD thesis, Wageningen University, The Netherlands, 2009
With summaries in English and Dutch

ISBN: 978-90-8585-506-4

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Enhancing relevant immunity of production animals to achieve more robust animals is receiving more and more attention. Several epidemics have hit production animals recently and with devastating consequences, but enhancing diseases resistance increasingly provides new opportunities. Furthermore, welfare and health of production animals is becoming a more and more consumer driven topic. Several routes are being used to approach the possibility of enhancing immunity such as selective breeding, enriched and altered housing conditions, vaccination programs, diet supplementation with immune stimulating components, and other management procedures. Disease susceptibility has been shown to be related to stress reactivity, which in turn is related to differences in HPA axis reactivity. Interestingly, independent of selection criteria used, the extremes of various selection procedures result in a recurrent dichotomy in HPA axis reactivity, either being hyperresponsive or hyporesponsive to stress. Animals with a hyperresponsive HPA axis show greater environmental sensitivity, while the hyporeactive animals are more intrinsically regulated. Often, research on immunomodulation is performed with compromised animals and/or exaggerated supplementation of dietary components in one generation of animals, but epigenetics by definition seems to be the mechanism for mothers to prepare their offspring for the environment they will be born into. Enhancing immunity through normal diet in uncompromised animals is rarely investigated, let alone over generations. In this thesis the aim was to induce immunomodulation through diet in selection lines of chicken that have previously been selected on their antibody response to sheep red blood cells over two generations of chicken. First, potential HPA axis differences were examined in these selection lines to establish their environmental sensitivity, whereafter immunomodulation through normal diet was investigated in humoral and cellular parameters of immunity. As humoral immunocompentence was not easily modulated, an immune trigger was used to detect potential differences in humoral reactivity. The selection lines showed differential sensitivity to immunomodulation by diet in both generations, suggesting that adaptation to environmental factors may be a line-specific (genetically based) process, with differential neuroendocrine regulation. Most interestingly, the second generation showed effects of the diets in all the selection lines, albeit in different manners. It is concluded that normal diet can cause immunomodulation, mainly in animals with hyper HPA axis reactivity, and that introducing such practices may be more beneficial when mothers are treated, as all offspring showed immunomodulation, irrespective of selection

line. While genetic background and/or epigenetic processes on neuroendocrine and immune regulation of the individual form the framework wherein individual immunomodulation by diet can take place, environmental conditions determine if the modulation is beneficial or not.

Voor Rob en Emma, mijn spetters.
xxx RAT

CHAPTER

I

General introduction

Immune system

Within a population there is great genotypical and phenotypical diversity in animals and how they function in response to ever changing environmental conditions. During an epidemic there are always at least a few individuals that will survive thanks to this large diversity in the individual animals. This phenomenon characterises the immune system well, as a large diversity in immune competence is necessary within a population ensuring its survival to lethal threats. The primary tasks of the immune system is to protect an organism from potentially harmful infiltration of pathogens from the outside world, and life threatening unregulated processes (e.g. tumor growth) in the internal environment (Mak, 2003). The first is reflected in the locations of the immune system: the mucosal immune system (the skin and all mucosa that come in contact with the environment, e.g. lung, intestine etc.) and the systemic immune system (all non-mucosal tissues, lymph network and blood vessels). Furthermore, the immune system can act very specifically within an organism, with in some cases even opposite reactions depending on where the immune response is occurring and can be measured. This is necessary because if an animal is e.g. wounded, it is detrimental for the organism as a whole when the immune system would act systemically. In such cases only the wound requires specific infiltration of immune cells and an immune response and not the organism as a whole.

The immune system is comprised of two systems that interact and communicate with each other continuously, the innate immune system and the adaptive or specific immune system (Mak, 2003; Calder, 2007). The innate immune system is represented by cells and humoral factors that have a broad specificity with germ-line encoded receptors and usually a fast response (seconds – hours) with apparently no memory of a prior exposure. The specific immune

Table 1. Immune System characteristics

Innate Immunity	Specific Immunity
Receptors have broad specificity (binding PAMP)	Receptors have very narrow specificity (binding specific epitopes)
Immediate response	Slow response
No memory of prior exposure	Memory of prior exposure

system is represented by cells and humoral factors with very narrow specificity Due to rearranged receptor genes, a relatively slow response (days) and with memory to the specific antigen. In addition, specific immunity is prone to maturation, whereas this is not the case for the innate responses.

The above may suggest that the immune system is very simple in organization, but while the understanding of the immune system grows, it becomes clear that this is all but true (Figure 1). Many cell types present in the immune system can be found in several different stages of maturation or differentiation, and several sub-types of cells, molecules and receptors are being found as more and more sophisticated techniques evolve. The last has major consequences on our understanding of the immune system. The stage of maturation or differentiation of, for example, the dendritic cell (DC), strongly determines the differentiation of various T-cell subtypes (Noverr and Huffnagle, 2005).

Innate immune response

On host cells, Pathogen Associated Molecular Patterns (PAMP) expressed on infectious bacteria, viruses and parasites may meet a number of pattern-recognition receptors (PRR) (Mak, 2003; Barton, 2008). Such receptors were present early in evolution and remained present by selection due to the presence of invasive micro-organisms at the population level. PRR are strategically expressed on cells which are first to encounter pathogens during infection: epithelial cells and effector cells of the innate immune system (dendritic cells and macrophages)(Berczi et al, 1998; Van Vliet et al, 2007). Currently, different serum proteins, including C-reactive protein (CRP), mannose-binding lectin (MBL) and LPS-binding protein, were shown to represent PRR. Moreover, PAMP upon binding to PRR induce in their target cells expression of several proinflammatory cytokines, like tumor necrosis factor (TNF)-α, IL-6, IL-12, IL-15, and type-1 interferons. Subsequently, inflammatory responses are modulated by the release of IL-10 and transforming growth factor (TGF)-β. In addition, pathogens will be bound to specific receptors expressed on leukocytes, especially when these pathogens are 'opsonized' by ligand molecules, like complement factors (C3), heat-shock proteins (hsp), LPS-binding proteins, etc. Such receptors include TOLL-like receptors (TLR) and these comprise now more than 10 family members. TLR belong to the class of PRR (Netea et al, 2005; Barton, 2008). These TLR are specific for structures present on bacteria, viruses and parasites and enable cells of the innate immune system which express these TLR

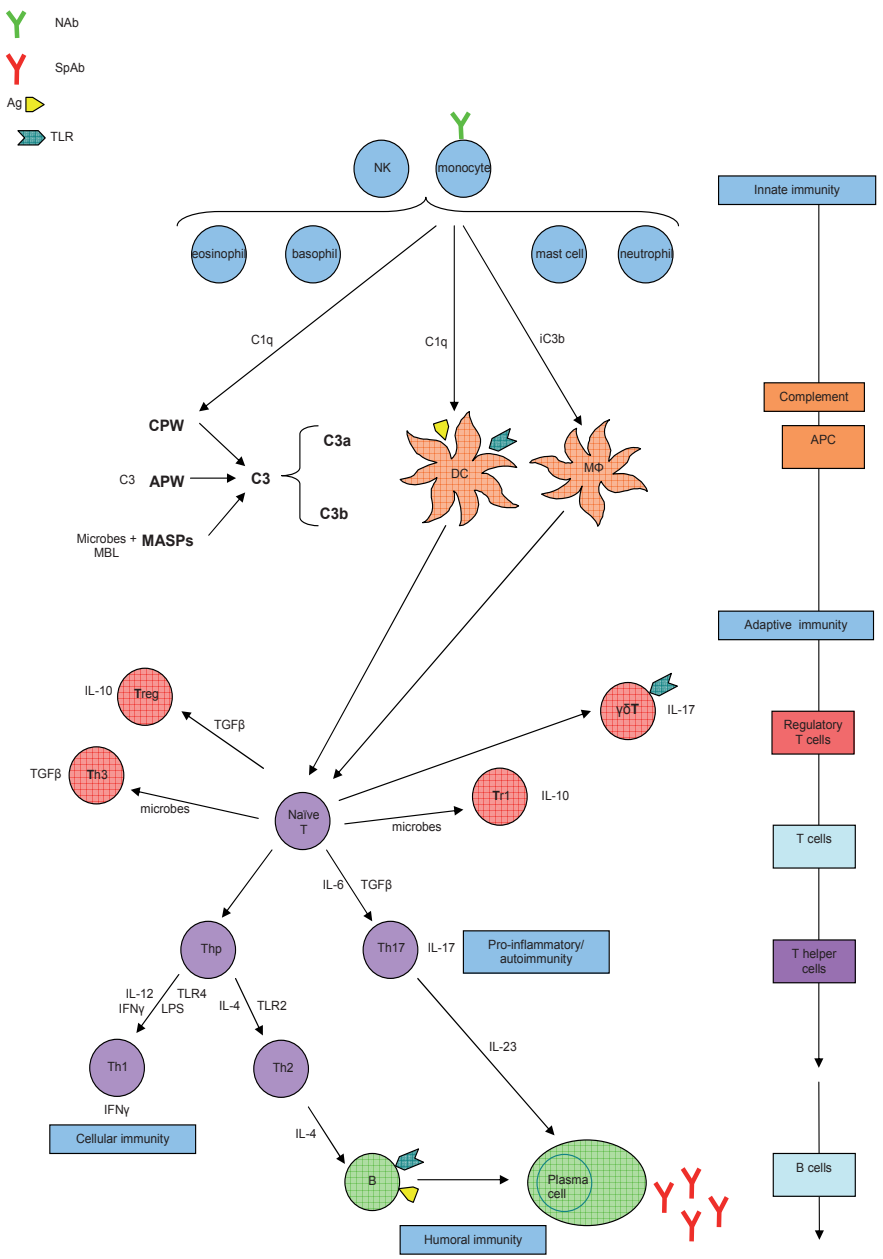


Figure 1. T-cell subsets and the Immune System

to react rapidly to these infections. By recognizing the PAMP by the TLR this pathogen-TLR interaction plays a decisive role in the rapid non-inflammatory clearance of infectious micro-organisms (Xu et al, 2004). Antigens, like bacteria can be opsonized later on during the immune response with antigen-specific antibodies or complement factors. This opsonization facilitates their phagocytosis by macrophages and their subsequent intracellular killing and degradation. Binding of PAMP to relevant PRR will thus result in production of pro-inflammatory cytokines, like IL-12 (Barton, 2008). It is becoming increasingly clear that recognition of conserved microbial or viral antigens via 'non-specific' receptors, e.g. TLR, heat-shock proteins (HsP) or complement component C3 receptors present on cells of the innate immune system (natural killer (NK) cells, phagocytes), or recognition of microbial antigens by natural antibodies (NAbs) form the first and probably most important defense mechanism against infection.

In recent years a lot of progress has been made in understanding how the innate immune system senses "danger signals" from microbes via PAMP and PPR (Köhl, 2006; Barton, 2008). Natural antibodies are antibodies present without (intentional) induction by means of e.g. vaccination. These antibodies can be produced spontaneously by B1 cells or after contact to cell wall fragments of autologous gut bacteria. They do not require T cell help and are considered to be part of the innate immune system more than of the adaptive immune system (Chou et al, 2008).

Sensing the pathogen results in the local induction of number of immune molecules/mechanisms. E.g. inducible Nitric oxide synthase (iNOS) is upregulated in local macrophages and iNOS expression results in the production of nitric oxide (NO) which is a key component in the antibacterial activity of macrophages. The complement system is composed of a series of blood proteins which interact as an enzyme cascade and function as a component of the acute inflammatory response. Several different complement pathways are distinguished. Activation of the complement system can result in direct killing of the pathogen or enhanced killing by other immune mechanisms. The "classical" complement pathway requires antibodies bound to a microbe. The other two pathways (the "mannose" and "alternative" pathways) can interact directly with microbes (Molina, 2004; Abbas et al, 2009).

After the invading pathogen has been detected (sensed) by the immune system of the host, signaling occurs that will result in the recruitment of effector molecules and cells from the blood to the site of infection. The intensity of the inflammation is dependent on the kind of pathogen and its quantity (Han and Ulevitch, 2005; Barton, 2008). In other words, if the resident and induced immune mechanisms have been sufficient to control the pathogen, the inflamma-

tory response will be less or remain absent. If on the other hand the pathogen is expanding, an inflammatory response will occur. This will greatly enhance the ability of the host to destroy the pathogen, but may result in (some) damage to the host tissues (collateral damage).

Adaptive immune response

T cells are divided into two classes: Cytotoxic T (Tc) cells and T helper (Th) cells. Naive CD4+ helper T cells (Th) develop into functionally mature effector cells upon stimulation with relevant antigenic peptides presented by major histocompatibility complex (MHC) class-II molecules on antigen-presenting cells (APC) (Netea et al, 2005). Based on the characteristic set of cytokines produced, Th cells are commonly segregated into at least two different subpopulations: Th1 cells producing exclusively interleukin-2 (IL-2), interferon-gamma (IFN-γ) and lymphotoxin. Th2 cells, on the other hand, produce IL-4, IL-5, IL-6, IL-10 and IL-13. These Th1 and Th2 subsets appear to be extremes in cytokine production profiles, and within these polarized subsets, individual Th cells exhibit differential rather than coordinated cytokine gene expression. The Th-1 and Th-2 subsets appear to cross-regulate each other's cytokine production profiles, mainly through IFN-γ and IL-10. IL-12 is a dominant factor promoting Th1 differentiation, and is produced by dendritic cells and macrophages. Polarization of Th2 cells is critically dependent on the presence of IL-4 produced by Th cells, basophiles and mast cells. APC-derived IL-6 has also been shown to induce small amounts of IL-4 in developing Th cells. IL-10 and APC-derived prostaglandin E2 (PGE2) inhibit IL-12 production and Th1 priming. These Th1 and Th2 subsets are the extremes in a whole set of intermediately polarized Th subsets (Chen and O'Shea, 2007).

Regulatory T cells, on the other hand, mediate active suppression of various immune responses (Chen and O'Shea, 2007; Afzali et al, 2007). These T cells comprise classical Th2 cells, capable of inhibiting Th1 responses, but also alternative T-cell populations. To complete the currently known T cell subsets section, recently, a novel Th subset has been described: the Th17 cells. These cells are probably induced by the presence of IL-23, and they produce IL-17 and IL-22 that both evoke inflammation (Bi et al, 2007; Stockinger et al, 2007; Jarnicki et al, 2008). In summary, an overview of the several cellular subsets comprising the immune response and its regulatory interactions are schematically visualized in Figure 1.

Innate versus adaptive immunity and disease resistance

Recognition of conserved microbial antigens via 'nonspecific' receptors (TLR, HsPs, complement factor C3) that are mainly present on cells of the innate immune system (NK cells, phagocytes), or recognition of microbial antigens by NAb form the first and probably most important defense mechanism against infection (Barton, 2008). In addition, activation of the innate immune response is a prerequisite for an appropriate specific immune response. Young animals are usually more sensitive to infectious diseases and this enhanced sensitivity might be related with an immature immune system, although all (cellular) components of the innate and specific immune systems are usually present in the young animal. Immaturity of the neonatal immune system may rest on various mechanisms such as decreased expression of surface markers (receptors) on immune cells, decreased production of cytokines, lower levels of complement components, and lower levels of NAb (Calder et al, 2006). An immature innate immune system will be less able to activate the specific immune response. The low level of NAb in young animals might also be related with the constitution of the intestinal flora, i.e. a low stimulation by that flora, and/or heritable levels of NAb and specific antibody production, respectively. It is reasonable to suggest that the maturation of the cellular part of the innate immune system (NK cells, phagocytes) depend on analogous microbial stimuli derived from the intestinal environment (Noverr and Huffnagle, 2005; Iweala and Nagler, 2006). In conclusion, there is an intricate balance in the gut between the endogenous flora in the one hand, and constituents of the innate immune system on the other hand. Disturbance of one of these two actors results in infection, inflammation and/or autoimmunity. Modulation of the innate immune system via the regulation of the intestinal microflora may add to the maturation of the innate immune system and in addition the maturation of the specific immune system (Hrncir et al, 2008).

Immunomodulation by diet

The influence of nutrition on the health status of animals is increasingly considered to be important in providing humans with a safe and sustainable source of food (Calder et al, 2006; Magginni et al, 2007; Fernandes, 2008). The concept of health status, particularly of farm animals, implies control of several different variables: including welfare, (chronic) stress, infectious pressures, endocrine status, immune status, a.o.i.e. resulting in constant and high-end production parameters. The housing and feeding conditions of animals thereby provide

important influences which exert their action by modulating the expression of the genetic background of the animal. As a result such animals display variable phenotypes having consequences for their "health status" and production parameters (McClure, 2008). This variability is further increased due to the fact that at neonatal age the immune system is not fully developed yet. In particular, the important T-cell immune system (including the development of functional T-cell subsets and the cytokines they produce) which induces and regulates important processes during an immune response (humoral and cell–mediated protective immune response) is not yet fully active (Forchielli and Walker, 2005).

It is thus necessary to be able to express the immune status, in a qualitative and quantitative fashion. This would preferably mean receiving information over the potency of the animal to adapt to altered environmental conditions by an optimized immune response (Albers et al, 2005; Wintergerst et al, 2007). In particular the nutritional constituents need to be evaluated for this capacity. Increased resistance in individual production animals will result in small cumulative evolutionary effects per generation resulting in more resistant animals during an infectious outbreak. Moreover, this genetic drift will result in increased heterogeneity in disease susceptibility among animals. High productive production breeds are generally selected for increased performance combined with genetic homogeneity, compromising the ability of the herd to survive life-threatening infections or any other stress-related equilibrium disturbance. Natural animal populations are inherently genetically heterogeneous thereby ensuring (at the expense of several individuals) to effectively survive exogenous threats (Coe et al, 2008). The herd is, due to its genetical heterogeneity, able to survive multiple disturbances and return to homeostasis because there are always animals that are able to deal with an individual disturbance. Collectively, the herd can be considered robust, even if individual animals are not, a condition generally occurring in outbred natural populations. During evolution animals and infectious organisms have co-evolved over many generations. As a consequence animals resistant to all infectious agents do not exist. In addition, despite the available evidence there is always the risk that increased resistance of the herd may result in increasing virulence of the virus. During such an outbreak, however, more animals will show subclinical infection complicating rapid and early detection of the infection on a population level. This dilemma illustrates the different demands for increased disease resistance for individual animals while on the population level susceptible animals will facilitate infection management and control.

To detect immunomodulation by diet, several approaches were used to determine which parameters may be suitable to use, starting with the report by Albers et al (2005) wherein suitability of immune parameters were classified in

several categories ranging from highly suitable to low suitability. Furthermore, literature was reviewed for parameters from other groups that had previously shown effects due to normal diets resulting in: i) effects on fertility and number of fetal deaths (Staiger, 1986); ii) effects on proliferation (Finamore et al, 2004); and iii) differences in IgG levels, vitamine E and rest (Lauridsen et al, 2007). Lastly, parameters from all compartments of the immune system were chosen that also reflected the chain of reaction during an immune response (i.e. from monocyte (upstream) > T-cell > B-cell > Ab production (downstream)). The cells of the innate immune system may be prone to an upstream modulation of the immune system, while e.g. effects on Ab production reflect downstream modulation of the immune system, although the latter may still be a reflection of upstream modulation as well.

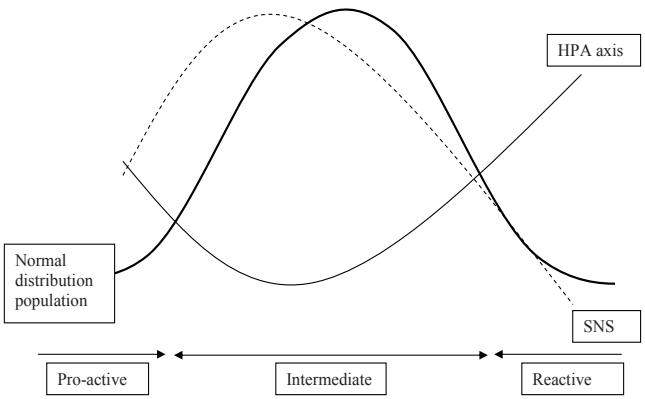
It is unlikely that a single protein by itself, clearly defines a specific treatment effect distinguishing it from all other diseases or conditions. Having several biomarkers may allow a more definite immune responsiveness, better disease stratification and additional value. However, it has been suggested that a large number of correlated biomarkers (although underscoring the importance of a specific biological pathway) is substantially less informative and predictive than a small number of uncorrelated biomarkers. In addition, when considering the importance of specific biological pathways, it is important to note that the combination of immunological data with physiological parameters, using correlation analysis, will provide clearer insights into the mechanisms by which these biomarkers regulate metabolic processes and ultimately change phenotypes.

Individual differences

When trying to understand the individual differences of animals in reactivity or susceptibility of e.g. behaviour, neuroendocrine system, immune system, maybe even differences in food or nutrient effects, the use of selection lines and defining general characteristics within these lines can be a useful tool. As reviewed by Koolhaas (1999) and Ellenbroek (2002) there are many similarities in most rodent selection lines that are used to date. Overall the main distinction that is made is between two extremes, the *proactive* animals and the *reactive* animals (Figure 2, overview of coping style characteristics). The proactive animals are characterized as more rigid and intrinsically regulated: strong routine formation in behaviour with a highly reactive sympathetic nervous system; the reactive animals are characterized as more flexible and regulated by environment: less active behaviour with higher HPA-axis and parasympathetic reactivity (Koolhaas

et al, 2007). These differences between the coping styles are found in stress paradigms where the opportunity is given to exibit this behavioural dichotomy; stress paradigms without such opportunities (such as inescapable footshock) may show comparable HPA axis reactivity.

On the level of the population, when regarding this dichotomy, the two extremes fulfil different needs in the survival of the population, the proactive animals are high in the social hierarchy and function best under such stable conditions as is seen in a well established population. The reactive animals are more open to environmental stimuli, are low in the social hierarchy and function best when emigrating from the established colonies and when new colonies are



Proactive		**Reactive**
Intrinsically regulated		Environmentally sensitive
Low turnover	DA/5-HT	High turnover
Fight/flight	Stress	Freeze
SNS	CNS	HPA axis & PNS
CA, NPY, opioids	Hormones	Corticosteriods/Ach
Th2	IS	Th1

Figure 2. Overview of coping style characteristics.

established (Koolhaas et al, 2007; Wolf et al, 2007, 2008). It becomes clear in this example that these differences are necessary and functional in the survival of a population during different and changing environmental conditions.

In the past decades differences in susceptibility to diseases of selection lines wherein a proactive/reactive strategy was found was often explained by dif-

ferences in the Th1/Th2 balance (Elenkov, 2008; Calcagni and Elenkov, 2006). In contrast, epidemiological research has shown that Th1 and Th2 type diseases coincide within a population (Sheikh et al, 2003). Since the recent discovery of the Th17 and the longer known Treg, it has become clear that such suggestions need to be adapted. For instance, Th17 has become the mayor player in diseases such as multiple sclerosis (MS, animal model EAE)(Aranami and Yamamura, 2008), ruling out the exclusivity of this disease to Th1 imbalance alone (Kavelaars et al, 1997). With the discovery of the regulatory T cells, the balance and interactions become even more complex (Figure 1). It becomes clear that not only e.g. the neuroendocrine balance at the adult age determines disease susceptibility but the road to adulthood as well (Koolhaas, 1999; Ellenbroek, 2002; Degen et al, 2004). The microbes that are encountered either through the digestive system or others, help form and develop the immune system but they also act on the neuroendocrine system (Hrncir et al, 2008). The same holds true for food nutrients and the accompanying food antigens, they too act on the immune system as well as on the neuroendocrine system. As imprinting periods are well known for (social) behaviour, the same mechanisms seem to be present for food nutrients and microbes irrespective of their affiliation (Spencer et al, 2006; Hrncir et al, 2008). Here too there seem to be periods in the developmental stages of organisms where these factors influence the developing adult so it most likely is best adapted to the environment wherein it is found. Interestingly, the mother also prepares her offspring perinatally for this particular environment through epigenetic mechanisms (Kuzawa, 2005; Grindstaff et al, 2006; Symonds et al, 2007; Darnaudrey and Maccari, 2008). The term epigenetics refers to changes in phenotype (appearance) or gene expression caused by mechanisms other than changes in the underlying DNA sequence. These changes may remain through cell divisions for the remainder of the cell's life and may also last for multiple generations. However, there is no change in the underlying DNA sequence of the organism; instead, non-genetic factors cause the organism's genes to behave (or "express themselves") differently (Jaenisch and Bird, 2003). The molecular basis of epigenetics is complex. It involves modifications of the activation of certain genes, but not the basic structure of DNA. Additionally, the chromatin proteins associated with DNA may be activated or silenced. This accounts for why the differentiated cells in a multi-cellular organism express only the genes that are necessary for their own activity. Epigenetic changes are preserved when cells divide. Most epigenetic changes only occur within the course of one individual organism's lifetime, but some epigenetic changes are inherited from one generation to the next (Chandler, 2007).

The interaction/cross-talk between the immune system and the neuroen-

docrine system is well established. The interactions between the two systems are accomplished by several mechanisms: 1) innervation of sympathetic nerves in organs of the immune system such as spleen, thymus, and bone marrow; 2) receptors on immune cells for hormones such as the catecholamines (epinephrine (EPI), norepinephrine (NOR) and dopamine(DA)), serotonin (5-HT), vasopressin AVP), neuropeptide Y (NPY), acethylcholine (Ach), endorphin (E) and glucocorticoids (GC) (Kin and Sanders, 2006; Meredith et al, 2005; Pállinger and Csaba, 2008; Wrona, 2006; Wheway et al, 2007). The expression of such receptors may be exclusive for immune cells while still present in these organs and disappear when systemically found. Furthermore, the effects on stimulation of the receptor may be inhibitory or enhancing depending on the cell type investigated (Kavelaars et al, 2002). Also the state of differentiation of the cell determines if the receptors are expressed or not, e.g. immature cells still in the thymus do express these receptors, but after maturation and when present in the periphery receptor expression is lost. Interestingly, monocytes and neutrophils seem to possess (almost) all hormone receptors. Receptor expression has been examined mostly in DC, monocytes/macrophages, neutrophils, granulocytes and lymphocytes (T- and B-cells). To date little is known about the hormone receptor expression on lymphocyte subtypes, except for adrenoreceptor (AR) expression which has also been determined on Th1 and Th2 subtypes (Nance et al, 2007). Th1 cells do express the β2-AR, while Th2 cells show no AR expression and α-AR are only found in cells present in lymphoid organs, while PBMC do not express α-AR under normal conditions (Kavelaars et al, 2002).

Genetics and the used animal model

For various reasons chicken provide a suitable animal model in studying immunomodulation of the immune response. Chicken are relatively more dependent on their innate defence mechanism for their health status compared to mammals, while they contain all the relevant immune cells and important molecules of the immune system alike these of mammals. Precisely the connection between the induction of a rapidly reacting innate immune response and the slower developing, but more efficient adaptive immune response can be studied very well in chicken. Up to date most research is done in compromised systems (e.g. nutritional deficiencies, diseased systems), here we will be investigating healthy uncompromised animals.

To examine the interplay between genetic background and environment chicken lines selected for the primary antibody response to Sheep Red Blood

Cells (SRBC) was used. Throughout 25 generations ISA Brown (Warren) medium heavy layers hens were divergently selected for their high or low primary antibody response to immunization with SRBC (Parmentier et al, 1994). From the same parental line a control line of unselected animals was kept. Unique to this selection line is that the birds from each line are not genetically identical (inbred) as is the case with most selection lines. The selection procedure is done such that the greatest amount of genetic variability is maintained within each line, keeping the normal genetic variance found in a population relatively intact. As a consequence birds with a low antibody response may still be found in the high line as well as the reverse.

Although the chickens have been selected for their primary antibody response to SRBC, in the last decennia it has become clear that other immune responses (e.g. cell mediated immunity, levels of natural antibodies, complement, etc.) have been affected by the selection as well (Parmentier et al, 1994, 1995, 1996, 2002, 2004; Kreukniet et al., 1996. There is evidence that neuroendocrine responsiveness has also been affected by the selection procedure (Siegel et al, 1992; Hangalapura et al, 2004).

Through interplay between the genetic background of an animal and the environment the immune system develops and matures. Innate immunity is already present at birth, the result of the genetic background of the foetus and maternal environment (phenotype). A case of somatic genetic memory is the immunological memory of the adaptive immune response in vertebrates. The immune system is capable of learning to recognize pathogens and keeping a memory of this learning process, which is the basis of the success of vaccinations. Antibody genes in B and T lymphocytes are assembled from separate gene segments, giving each lymphocyte a unique antibody coding sequence leading to the vast diversity of antibodies in the immune system. If stimulated by an antigen (e.g. following vaccination or an infection with a pathogen), these antibodies are further fine-tuned via hypermutation. Memory B cells capable of producing these antibodies form the basis for acquired immunological memory. Each individual therefore carries a unique genetic memory of its immune system's close encounters with pathogens. As a somatic memory, this is not passed on to the next generation. Lamarckian inheritance has been found within the immune system, which is specifically true for IgG and IgA, but not IgM. Here it has been concluded that maternal antibodies are important for an actively induced neonatal imprinting period (Lemke et al, 2004). Other processes determining phenotypic responsiveness, range from Mendelian inheritance to epigenetic controlled expression of genetic loci (Horton, 2005; Champagnat et al, 2006). Breeding and selection for only a limited set of production parameters has potentially biased the genetically deter-

mined ability to mount a coordinated adaptive response to increasingly variable environmental factors. These factors include type of husbandry, infectious load, level of chronic stress, handling, transport and others. Animals thus have to cope with profound changes in their environment that occur at multiple time points during their life: hatching or birth, weaning, fasting, transport, indoor vs. outdoor housing, etc. These rapidly changing conditions provide stress factors of multiple kinds precipitating into increased disease susceptibility. Moreover, the robustness of these immune compromised animals to withstand rapid and extreme changes in the environment is decreased.

Immune competence vs. Immune responsiveness

Immune competence of animals can differ due to several factors as can be seen in the selection lines mentioned before. When considering the immune competence of the selection lines used throughout the experiments described here, the high line responders are considered more immune competent than the low line responders, as they always have the higher levels of e.g. SpAb's, Nab's etc (Star et al, 2007). Even though these selection lines show different levels of immune competence, their immune responsiveness (IR) can be the same, as is often seen in data expressed as the delta (IR-baseline) immune parameter.

Can higher immune competence or greater immune responsiveness be considered more beneficial? Never in nature this will be true in absolute terms. Especially in nature, there are always situations wherein the costs of one (or more) highly evolved organ system or responsiveness in some individuals can be in their (dis-)advantage under specific circumstances. Again, the effects of an epidemic makes this clear: only a small group will survive and which animals these are, the high immune competent or the low immune competent is totally dependent on the characteristics of the infectious agent and other environmental circumstances (e.g. famine, cold, etc.). This could be different for animals that are held under relatively controlled conditions, although generalizations in this area remain difficult.

Within neuroendocrine research of individual differences, it is known that baseline levels of hormones often do not differ between animals of different coping styles, while after (psychological or physiological) stressors, hormone levels do differ (De Boer et al, 2003; Veenema and Neumann, 2007). Individual differences, or coping styles are reflected in the reactivity of animals to either intrinsic and/or environmental cues, requiring by definition a trigger to make such differences measureable. This neuroendocrine approach was used in this thesis

with parameters of the immune system as read out.

Homeostasis versus allostasis

Originally, homeostasis was the term used for the organism that is adapted to its environment and therefore has a stable neuroendocrine, autonomous nervous system, immune system and shows "normal" behaviour. Specifically for the immune system, homeostasis is considered to be the moment when the immune system has recovered from an immune response and is back to a balanced level of all compartments. Homeostasis actually requires that there are no reactions to the environment of a organism, because this would imply that the organism is in a unstable state. Within the homeostasis model, stress is everything that disrupts the balance of any system in the organism and is therefore always considered to be bad.

In recent years the term allostasis is being used more and more wherein the stable and balanced organism is reached by change (Korte et al, 2005, 2007). Here, change and therefore a reaction to the environment has become a necessity for the organism to adapt to the environment it is living in. By changing the organism can adequately adapt, without change, there is a chance that the organism will not adapt with all possible consequences. In the allostasis model, "stress" has become a necessity to help the organism change as required by the environment.

Where in the past every disruption of the balanced organism was considered stress, within the allostasis model, "allostatic load" defines the amount of change or adaptation of the organ systems challenged without causing adverse effects. When the allostatic load for the animal is too great, the organism will not have the appropriate response to a stressor e.g. infection, and either the response is not sufficient, thus the animal becomes sick; or the response is too much meaning that the immune response was not adequately stopped. Furthermore, findings such as reduced hormone responses to a repeated stressor, fit within the allostasis model as allostasis suggests a form of learning. Therefore allostasis requires a response of the organism to e.g. environmental factors, but the amplitude of the response should be lower after re-exposure reflecting the level of adaptation, i.e. the lower the response after re-exposure the greater the level of adaptation or learning.

Adaptation to the environment requires systems that relay the environmental information to the organism. The sensory systems (taste, touch, sight, hearing, smell) represent the first line of contact/communication between an organism and its environment. Here we suggest that the immune system also acts as

a sensory system making interaction and adaptation of an organism to its environment possible (Blalock, 1984).

Aim of this project

The main aim of this project is to examine if a normal diet as environmental factor can modulate the immune response of healthy chickens genetically selected for their primary antibody response to srbc. Furthermore, the use of selection lines may facilitate the understanding of the investigated immune parameters in relation to the specific genetic background of the animals. Lastly, possible effects over generations (epigenetics) are examined.

1) By using the immune system as sensory organ, investigate if small nutritional differences in two complete layer feeds are detected by parameters of the immune system.

2) Determine immune parameters that best reflect immunomodulation by diet.

3) Examine the potential differences in immunity in relation to genetic background of the birds.

4) How the immune system is affected over generations.

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CHAPTER

II

Individual behavioral characteristics of wild-type rats predict susceptibility to experimental autoimmune encephalomyelitis

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Brain, Behavior and Immunity (1999) 13(4); 279-286

Abstract

Neuroendocrine–immune interactions are thought to be important in determining susceptibility to autoimmune disease. Animal studies have revealed that differences in susceptibility to experimental autoimmune encephalomyelitis (EAE) are related to reactivity in the hypothalamo–pituitary–adrenal axis. It is known that there is a close relation between neuroendocrine parameters and behavioral characteristics, suggesting that behavior and disease susceptibility may be associated. In the present study we investigated whether behavioral characteristics of wild-type rats are related to susceptibility to disease. We show here that the latency of the animal to attack an intruder correlates significantly with the EAE disease score: animals that do not attack the intruder during the test period are more resistant to the disease than animals with short attack latency times. These data, obtained in an unselected strain of wild-type rats, demonstrate that behavioral response patterns of individual animals can in part predict susceptibility to autoimmune disease.

Introduction

Over the past years, it has become evident from animal research that genetic differences in neuroendocrine responses are associated with differences in susceptibility to autoimmune diseases. The first data in this area are from Sternberg et al., who investigated the differences in susceptibility to streptococcal cell wall-induced arthritis (SCW) between Lewis and Fischer rats (Sternberg et al, 1989a). Lewis rats are highly susceptible to SCW arthritis and other autoimmune models, whereas Fischer rats are resistant. Investigation of the neuroendocrine reactivity in these two strains of rats demonstrated that the reactivity of the hypothalamo–pituitary–adrenal axis in Lewis rats is lower than in Fischer rats (Sternberg et al, 1989b; Sternberg et al., 1989a). These differences in reactivity were observed after activation of the HPA axis by immune stimuli, e.g., injection with SCW or LPS, as well as after exposure to a novel environment (Sternberg et al., 1989a).

Evidence for a causal relation between HPA axis activity and susceptibility to disease was provided by demonstrating that treatment of Fischer rats with a glucocorticoid antagonist transforms them into SCW arthritis susceptible animals, whereas supplementation of Lewis rats with a glucocorticoid renders these animals resistant to the disease (Sternberg et al., 1989a).

More recently, we have demonstrated that the association between neuroendocrine response pattern and susceptibility to autoimmunity is not restricted to Lewis and Fischer rats. We examined susceptibility to experimental autoimmune encephalomyelitis (EAE) in two lines of Wistar rats (APO-sus and APO-unsus) that were selected on a pharmacogenetic basis with sensitivity to the dopaminergic agonist apomorphine as the selection criterium (Cools et al, 1993; Cools et al, 1990). Our data demonstrated that APO-sus rats are resistant to EAE, whereas APO-unsus do get the disease. Similar differences in susceptibility to arthritis have been observed in these rats as well (Van de Langerijt et al, 1994).

Further analysis of the immune response in APO-unsus and APO-sus rats revealed that the difference in susceptibility to autoimmunity is related to a difference on the level of T helper-1/T helper-2 (Th1/Th2) cytokine balance between rats from the two strains (Kavelaars et al, 1997a). In APOunsus rats that are sensitive to Th1-dependent autoimmune diseases like arthritis and EAE, the ratio of Th1 over Th2 cytokines produced after ex vivo stimulation is higher than in APO-sus rats (Kavelaars et al., 1997a). The two lines of rats show a dichotomy in the reactivity of the neuroendocrine system on the level of the HPA axis. APOsus rats have higher plasma ACTH levels at rest and in response to a stressor. In addition, corticosterone responses are higher and last longer in APO-sus rats

than in APO-unsus rats (Rots et al, 1996; Rots et al, 1995).

It is well known in a wide variety of genetic selection lines that the differences in neuroendocrine response patterns are associated with differences in behavioral characteristics. For example, APO-sus Wistar rats that do not get EAE display higher locomotor responses in a novel environment than APO-unsus rats (Cools et al., 1993; Cools et al., 1990; Kavelaars et al., 1997). However, many strains and selection lines of laboratory rats are the product of rather intense breeding procedures and genetic selection processes. Therefore, one may wonder whether the observed relation among neuroendocrine response patterns, behavioral characteristics, and susceptibility to autoimmune disease is a more general phenomenon which can be observed in a normal population as well. To answer this question we used a strain of laboratory bred feral (wild-type) rats known to differ widely in their behavioral reactivity under a number of (social) environmental conditions. The attack latency in a resident–intruder paradigm was used as a reliable indicator of this individual differentiation in behavioral reactivity or coping style (Benus et al., 1998). The central question in the present study was whether behavioral characteristics may predict their susceptibility to autoimmunity. Animals were behaviorally characterized prior to inoculation with myelin basic protein emulsified in Freund's complete adjuvant for induction of EAE.

Materials and Methods

Animals

Conventional male wild-type rats (*Rattus norvegicus*) bred in the Department of Animal Physiology, Groningen, The Netherlands, were used in this study. Animals were housed in groups of five individuals from weaning until the start of the experiments (4 months of age) in clear macrolon cages (60 x 40 x 20 cm) at ambient temperature on a 12-h light/12-h dark cycle. Bedding consisted of wood shavings, and food and water were available *ad libitum*. All experiments were performed in accordance to international and institutional guidelines for animal care.

Determination of Attack Latency Time (ALT)

Wild-type males were housed together with a sterilized wild-type female in wooden cages (85 x 60 x 50 cm) for 2 weeks. After these 2 weeks (after temporary removal of the female), each male animal was exposed to an intruder (male Wistar rat of 250 ± 25 g) in a standard resident–intruder test (Koolhaas et al, 1980) on 4 consecutive days. The latency to first attack (in seconds) was determined and the intruder was removed immediately. When animals did not attack the intruder within 600 s, the test was ended by removal of the intruder. The ALT represents the average of the four daily trials.

Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalitis was induced in a total of 31 animals in two separate experiments. Data reflect combined results of these two experiments. One animal died after inoculation with myelin basic protein (MBP) in FCA.

Two weeks after the determination of the ALT, animals were inoculated in the hind paw with 100 ml of inoculate under brief halothane anesthesia. The inoculate consisted of 2 mg myelin basic protein isolated from guinea pig brain in 1 ml saline mixed with 1 ml complete Freund's adjuvant (Difco, Detroit, MI) to which 10 mg mycobacterium tuberculosis H37Ra was added. Rats were examined daily to score the development of clinical signs of the disease. Clinical signs were scored on a scale from 0 to 7: 0, no disease; 1, loss of tail tonicity; 2, paralysis tail; 3, hind paw weakness; 4, severe locomotor problems; 5, paralysis of both hind limbs; 6, paralysis up to diaphragm; 7, death as a result of EAE.

Determination of HPA Axis reactivity

One week after the determination of the ALT, animals were provided with

a silastic heart cannula (id 0.5 mm; od 0.9 mm; Dow Corning) through the right jugular vein under Hypnorm anesthesia (10 mg/kg fluanisone and 0.2 mg/kg fentanyl ip; Janssen Pharamceutica) after premedication with atropine (1mg/kg, ip) and diazepam (5 mg/kg ip). The canula was externalized on the top of the skull as described (Steffens, 1969) to allow frequent blood sampling in conscious, undisturbed freely moving rats (Wiersma and Kastelijn, 1985; Steffens, 1969).

After surgery, animals were allowed to recover for 10 days and habituated to the sampling procedure. Blood samples were taken at baseline (t = -10 min) and at 30, 60, 90, and 120 min after administration of CRH. CRH (500 ng/kg body wt) was administered iv via the canula. This dose of CRH has been used successfully to detect individual differences in HPA axis reactivity (Ruis et al, 1998; Buwalda et al, 1998).

Plasma corticosterone was determined by reversed-phase high-performance liquid chromatography (Dawson, et al., 1984).

Data Analysis

Data were analyzed by Spearman rank correlation test or ANOVA, and $p < 0.05$ was considered statistically significant.

Results

Behavioral Characteristics of Wild-Type Rats Used for Induction of EAE.

Animals were characterized behaviorally by determining the latency to attack an intruder in a standard resident–intruder test. In our test group, attack latency times determined as the average of four trials on consecutive days varied between 82 and 600 s. Nine of 31 animals did not attack the intruder in any of the four trials. For the other 22 animals median attack latency time was 362 s (range 82–567 s).

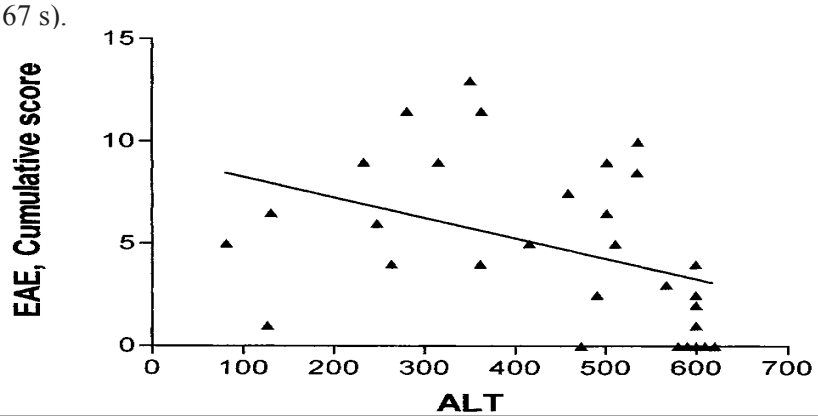

Figure 1. Relation between ALT and disease score. Attack latency times were determined using a standard resident–intruder test (see Methods). Two weeks after the last resident–intruder test, animals were inoculated with MBP in FCA in the hind paw. Clinical signs of disease were scored daily and total disease scores for each animal were determined by summation of the score of each day. Spearman rank correlation test for ALT 3 disease score: r = - 0.54, n = 30, two-tailed p = 0.002.

Relation between Disease Score and ALT.

To determine whether there is a relation between ALT and susceptibility to autoimmunity, all animals were inoculated with MBP in FCA 14 days after determination of ALT. Clinical symptoms of the disease were scored daily. First symptoms were observed on days 9 or 10 after inoculation. Maximal disease activity occurred on day 14, after which the animals gradually recovered. Total disease scores were determined by summation of the daily scores for each individual animal. The data in Fig. 1 demonstrate that there is a significant inverse relation between ALT and total disease score (two-tailed Spearman rank correlation test: r = - 0.54, n = 30, p = 0.002).

To determine whether the difference in total disease score is due to differences in the course of the disease, we also analyzed mean disease scores in two groups of animals: passive animals defined as animals with the maximal

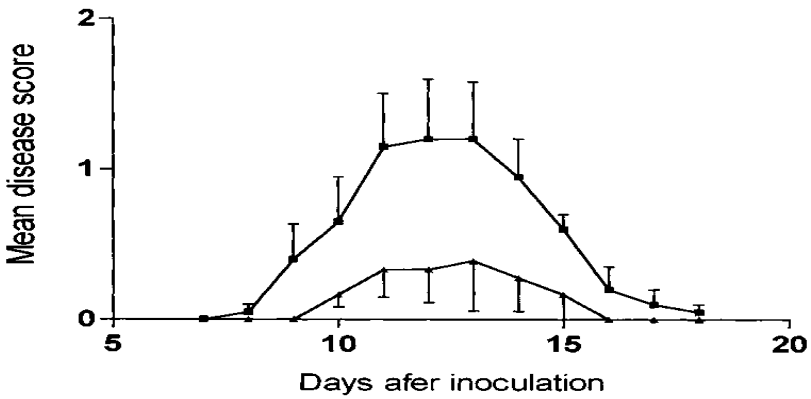

Figure 2. Course of EAE. Attack latency times were determined using a standard resident–intruder test and disease was induced as described in the legend to Fig. 1. Data represent mean disease scores for animals with maximal ALT (ALT 600 s, n = 9) (▲) and active animals with ALT in the lower 30th percentile (n = 10, ■). ANOVA ($F = 21.4$; $p < 0.001$).

ALT of 600 (n = 9) and active animals with ALT in the lower 30th percentile (n = 10). As depicted in Figure 2, there were no differences with respect to day of onset, peak or duration of the disease. However, disease severity did differ significantly between these two groups (ANOVA:group effect, $F(1,198) = 21.4$; $p<0.001$; time effect: $F(11,198) = 4.38$, $p<0.001$; Interaction effect: $F(11,198) = 1.15$; $p = 0.33$).

Reactivity of the HPA Axis.

In a second (new) group of animals, to determine whether ALT are related to the reactivity of the HPA axis we measured the corticosterone response to administration of CRH. Our data demonstrate that there was no correlation between ALT and corticosterone response determined as area under the curve (not shown). Moreover, there was no difference in the time course of the corticosterone response between animals with the maximal ALT and animals in the lower 30th percentile (Figure 3).

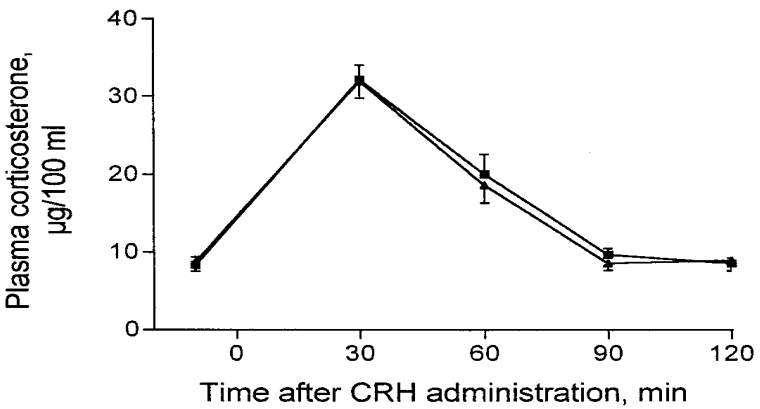

Figure 3. Corticosterone response to CRH. Attack latency times (ALT) were determined using a standard resident–intruder test. One week later, animals were provided with a heart cannula that was externalized on the skull to allow blood sampling. After 10 days recovery, blood samples were taken at the indicated time periods after iv administration of CRH (500 ng/kg body wt). Plasma corticosterone levels were determined. Data represent plasma corticosterone levels for animals with maximal ALT (ALT 600 s, n = 12) (▲) and active animals with ALT in the lower 30th percentile (n = 14, ■). ANOVA: $F(1,120) = 0.14$, $p = 0.71$.

Discussion

In an outbred strain of wild-type rats, we demonstrated that behavioral characteristics in a resident–intruder paradigm partially predict the severity of EAE in these rats. Our data show that aggressive rats (short ALT) are more susceptible to EAE than nonaggressive rats (ALT 600 s), who hardly get the disease. The ALT in a resident–intruder test is indicative for the level of aggressive behavior and coping style of the individual rat (Koolhaas and Bohus, 1995). Hence, in an unselected population of wild-type rats, the large individual susceptibility to autoimmune disease correlates with the individual capacity to cope with environmental challenges.

From previous research, we know that there is a strong correlation between the level of aggressive behavior in these wild-type rats and the neuroendocrine response to a number of stressors. Exposure of the animals to a defeat stress or to a shock prod results in sympathetic, adrenomedullary, and adrenocortical reactivity (Sgoifo et al, 1996). The reactivity of the sympathetic (plasma noradrenaline) and adrenomedullary (plasma adrenaline) system can be predicted on the basis of the ALT. In animals with short ALT, i.e., aggressive animals, stress-induced increases in plasma adrenaline and noradrenaline are much higher than in animals with ALT of 600 s (Sgoifo et al., 1996). However, the reactivity of the HPA axis determined as plasma corticosterone responses to either shock prod burying or defeat was not related to attack latency times. This finding is in line with our present data showing that there is no relation between ALT and corticosterone response after administration of CRH. We also have preliminary data suggesting that LPS-induced corticosterone responses are not related to ALT. Therefore, we conclude that the relation between ALT and EAE susceptibility is not simply a reflection of differences in HPA axis activity. These data are in contrast to what has been observed in Fischer versus Lewis rats or when comparing APO-sus and APO-unsus Wistar rats. In these strains or lines of rats, susceptibility to autoimmune disease coincides with blunted reactivity of the HPA axis.

If reactivity of the HPA axis is not related to disease susceptibility in the more aggressive rats, which other systems are involved? One possibility would be that the increased sympatho–adrenomedullary reactivity of the susceptible animals plays a role. Catecholamines can modulate the activity of the immune system in several ways. Lymphoid cells express a- and b-adrenergic receptors, triggering of which can result in alterations of cytokine production and thereby may contribute to disease activity (Khan et al., 1986; Bisphoric et al, 1980; Van der Poll et al, 1996; Heijnen et al, 1996; Spengler et al, 1990; Spengler et al, 1994). In general, β2-adrenergic receptor agonists will inhibit proinflammatory cytokine production (Van der Poll et al., 1996). However, recently we demonstrated that the production

of the proinflammatory cytokine IL-8 is enhanced by addition of a β2-adrenergic agonist (Kavelaars et al, 1997). Moreover, it has been shown that administration of the α1-adrenergic receptor antagonist prazosin results in decreased EAE symptoms (Brosnan et al, 1985). Thus, it is conceivable that higher levels of noradrenaline enhance disease symptoms, probably via α-adrenergic receptors giving rise to upregulation of proinflammatory cytokines (Khan et al., 1986; Bisphoric et al, 1980; Van der Poll et al., 1996). This interpretation may be supported by recent evidence that the differential sympathetic reactivity in the wild-type rat is partially due to a less functional presynaptic and postsynaptic β2-adrenergic receptor in the nonaggressive male (Smit et al, 1998).

In summary, we have demonstrated that behavioral characteristics in wild-type rats correlate with susceptibility to EAE. In these animals, there is no evidence for a relation between reactivity of the HPA axis and disease. Further research would be needed to determine to what extent the differential EAE susceptibility might be explained by the differential reactivity of the sympatho–adrenomedullary system. The possibility should be considered, however, that more than one neuroendocrine pathway will contribute to the susceptibility to autoimmune disease.

Acknowledgements

This study was supported in part (R.T. and J.B.) by NWO grant 904-64-021.

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CHAPTER

III

Chicken lines selected for their PrimaryAntibody response to SRBC show differential HPA axis responsiveness to mild stressors

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Poultry Science (2009) 88; 1879-1882

Abstract

The interaction between the neuroendocrine system and the immune system is well established and support their mutually affecting relationship. Many animal selection lines have been created according to individual behavioral or neuroendocrine responses to stress. Here we present two chicken lines selected for 25 generations for their primary antibody response to immunization with sheep red blood cells (SRBC), as well as the control line from the same parental strain. In the first experiment, the blood sampling procedure caused a mild stress response, with the expected increase in plasma corticosterone levels. In a second experiment, group housing caused the expected increase in corticosterone levels. In both experiments the hens of the Low line showed the greatest increase in corticosterone levels to our two mild stressors. Our results show that birds selected through out 25 generations for an immune parameter show different HPA axis responsiveness.

Introduction

It is widely accepted that there is a 2-directional relationship between the neuroendocrine system and the immune system, and the complexity of this relationship is still being unraveled (Beishuizen and Thijs, 2004). The genetic background and environmental factors determine the phenotype of the individual (Ellenbroek and Cools, 2002). Within this interplay, there is a profound and complex relationship, which is reflected in stress handling and disease resistance in the individual (Elenkov and Chrousos, 2006). The influence of genetic background on disease resistance is often investigated in animal model species using selection lines that are created based on different behavior in experimental settings or on different endocrine responses to stress, or both (Lindqvist et al., 2007; Owen et al., 2008). The extremes of such selection lines have shown similarities in typical behavior and their endocrine responses that have been translated into 2 different coping styles. Animals showing a *proactive* coping style show active behavior when given this possibility and a low hypothalamic-pituitary-adrenal (HPA) axis response as the proactive behavior requires the activation of the sympatho-adrenomedullary system. On the other hand, the animals showing a more passive style of coping, also known as *reactive* coping, show less behavioral activity with a high HPA axis response. This dichotomy has been found in many species, such as rodents, chicken, and fish (Koolhaas et al., 2007; Øverli et al., 2007).

Disease resistance has been postulated to be a multigenic trait, regulated by the immune system and its modulations by interactions with physiological and environmental factors (Zekarias et al., 2002; Fulton, 2004; Owen et al., 2008). Here we present a selection line of layer hens that has been selected for 25 generations for their primary antibody (Ab) response to immunization with SRBC. Three lines were selected: the high responders, which produce high Ab levels to SRBC after immunization; the low responders, which produce low Ab levels to SRBC after immunization; and a control line from the same parental stock (Parmentier et al., 1994). In most selection lines, the lines are inbred so there is genetic uniformity. In the used selection lines, the selection is done randomly, keeping the maximum genetic variation intact, thereby causing the lines not to be exclusive to the selection criteria. For example, within the group of high responders, birds can be found that still show a low specific Ab formation and vice versa in the group of low responders. Interestingly, almost all immunological parameters of these lines differ, including various parameters of the innate and adaptive immune systems as well as humoral and cellular immune parameters (Parmentier et al., 1994, 1995, 1996, 2002, 2004; Kreukniet et al.,

1996).

Recently, neuroendocrine differences in responsiveness were found in these selection lines in response to cold stress (Hangalapura et al., 2004), suggesting a difference in HPA axis reactivity. Siegel et al. (1992) presented the first differences in corticosterone responses to immunization in these birds. Here we present data in which the differences in immune competence appear to be related to the same dichotomy as is seen in selection lines based on behavioral or endocrine criteria, or both.

Materials and Methods

Chickens, Housing, and Feed

In this experiment, ISA Brown (Warren) medium heavy layer hens from 3 lines were used. The selection lines were selected for 25 generations before this experiment for either high or low primary Ab responses at 5 d after i.m. immunization (without the use of adjuvant) with SRBC at the age of 35 d. The randomly bred third line is the control line, originating from the same parental stock. The first experimental group consisted of 72 hens evenly divided over the 3 selection lines, but not immunized with SRBC. The second experimental group consisted of 150 hens evenly divided over the 3 selection lines and without immunization with SBRC.

The birds of the first experiment were kept according to routine procedures for layer hens in individual battery cages. The hens of the second experimental group were kept in a floor housing system with a total of 6 birds per cage, 2 birds of each line. The light regimen was 14 h of light (0500 to 1900 h), and temperatures were between 16 and 21°C. The birds were fed *ad libitum* with a commercial diet (180 g/kg of CP, 3,460 kcal/kg) with free access to water. Birds were weighed weekly, before the sampling procedure, therefore making them accustomed to handling.

All experiments were approved by the Animal Welfare Committee of Wageningen University.

Blood Sampling and Corticosterone Determination

All blood samples were taken by wing venipuncture between 0800 and 1000 h, and the sampling procedure took a maximum of 2 min per bird. The selection lines were sampled at random so all lines were sampled evenly over the whole sampling period. Blood samples of the first experimental group were taken at 9 wk of age, and the sampling procedure was considered to be comparable to a mild stressor (Webb and Mashaly, 1984). The second experimental group was group-housed, which has previously been shown to elevate corticosterone levels, and was sampled at the age of 8 wk (Mashaly et al., 1984). Plasma from heparinized blood was collected after centrifugation of the samples and then stored at −20°C until further analysis. Plasma corticosterone was determined using a RIA kit (IDS Inc., Bolton, UK).

Statistical Analysis

All data not normally distributed were omitted from analysis. The mean differences due to blood sampling and line were tested with Bonferroni's

correction. Differences between the selection lines were determined by multiple comparison of the means. All data are presented as mean ± SEM. All analyses were according to SAS procedures (SAS Institute, 2003).

Results and Discussion

In the present study, the modulatory effects of 2 mild stressors, a standard blood sampling procedure and social housing, were examined in 2 selection lines of chicken, together with the control line, on the reactivity of the HPA axis as measured by the plasma corticosterone levels.

The selection lines showed differential corticosterone responses to the blood-drawing procedure as is presented in Figure 1. The variance of the corticosterone levels increases, respectively, from high, control, to low line hens, with the low line showing the greatest variance. The high and control line birds showed comparable mean corticosterone levels, and the low line birds showed the highest mean levels of plasma corticosterone (Table 1). The differences in corticosterone levels between the low line birds and the control and high line and low line birds were significant.

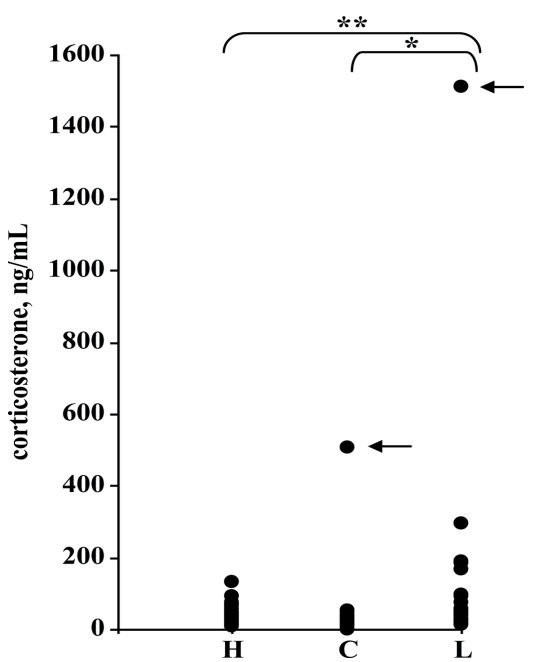

Figure 1. Scatter plot of the measured corticosterone levels (ng/mL) in plasma in all of the individual birds of the first experiment with the blood-drawing procedure as mild stressor in the high (H), control (C), and low (L) line hens. n = 23 to 24 birds per line. Arrows indicate data omitted from the analysis. *P < 0.05; **P < 0.01.

The variance in corticosterone levels was similar between the selection lines in the second experimental group (Figure 2). The data obtained from the second experimental group, with social housing plus blood drawing as mild stressor, showed significant differences between the low line birds when compared with the control and high line birds (Table 1).

To date, the neuroendocrine relationship with the immune system has mostly been examined in selection lines based on either behavioral or neuroendocrine criteria, or both (Owen et al., 2008). Here, we examined the HPA axis responses to 2 mild stressors (the bloodsampling procedure and social housing coupled with blood sampling) in a selection line of chicken based on an immunological criteria,

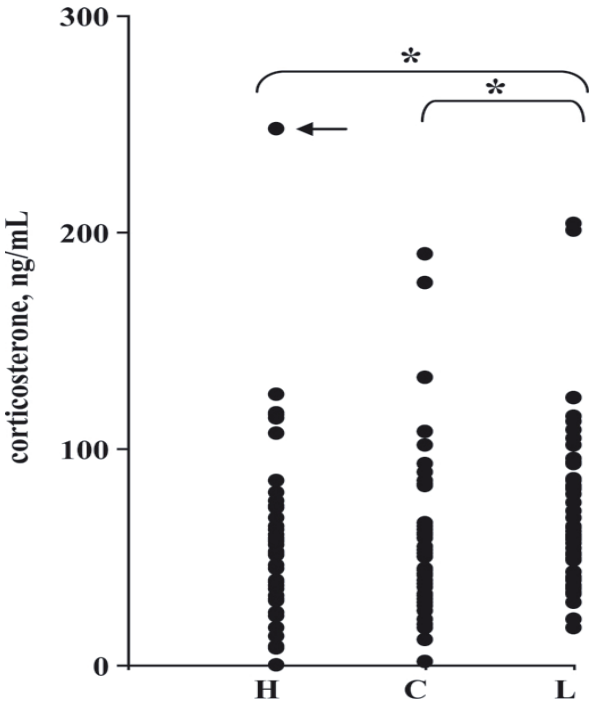

Figure 2. Scatter plot of the measured corticosterone levels (ng/mL) in plasma in of all the individual birds of the second experiment with the blood drawing and social housing as mild stressor in the high (H), control (C), and low (L) line hens. n = 46 to 50 birds per line. Arrow indicates data omitted from the analysis. *P < 0.05.

the primary Ab response to SRBC. In this experiment, it was of crucial importance that we used noninbred divergently selected chicken lines, causing larger variance in the measured results as is comparable to neuroendocrine measurements of

outbred animals (de Boer et al., 2003). The corticosterone response to the sampling procedure was different between the lines, wherein the low line birds with a low Ab response showed the highest plasma corticosterone levels in both experimental settings. Because the birds were accustomed to weekly handling, the sampling procedure was considered to be comparable to a mild stressor. Mild stress only elevates corticosterone levels slightly (Salomé et al., 2006; Hazard et al., 2008).

Table 1. Measured average corticosterone levels1 (ng/mL) in both experimental groups from the high, control, and low line hens

Line[1]	Blood sampling	Group housing
High line (H)	46.1	57.8
Control line (C)	28.2	57.7
Low line (L)	81.3	76.3
SEM	12.2	6.5
H vs. L[3]	0.0286	0.0437
C vs. L	0.0060	0.0457
Line	0.0144	0.0658

[1]Data are group (least squares) mean: blood sampling n = 22 birds per line; group housing n = 44 to 49 birds per line.
[2]Line = line effect.
[3]Line contrasts.

Our data together with the data from Siegel et al. (1992) and Hangalapura et al. (2004) showed that the low line birds have increased HPA axis reactivity in different experimental settings and when compared with the high line and control line birds. The measured corticosterone responses during cold stress and immunization clearly showed that the high line birds showed a rapid and lower corticosterone response. These results indicate that although selected for an immune trait, these selection lines show differential sensitivity to different stressors as is well known in coping styles. Within coping styles, the reactive animals are more environmentally sensitive and the proactive animals are more intrinsically regulated (Koolhaas, 2008). In the previously discussed context of coping styles, our data suggest that the low line birds may be considered to be reactive copers and the high line birds may be considered to be proactive copers. Therefore, selection based on immunological criteria results in the same dichotomy as is seen with selection based on either behavioral or neuroendocrine differences (van Hierden et al., 2002; Flisikowski et al., 2008).

In conclusion, chickens selected for their differential Ab response show the same dichotomy in HPA axis responsiveness as is seen within coping styles. Because these selection lines have already been extensively investigated for various immune traits, they present a suitable model for investigating immune traits and coping styles (i.e., stress reactivity and disease resistance).

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CHAPTER

IV

Mild stress due to blood sampling alters immune parameters in chicken lines with different HPA axis reactivity in a line-specific manner

R.Adriaansen-Tennekes, H.K.Parmentier, and H.F.J.Savelkoul

Concept

Abstract

The interaction between the neuroendocrine system and the immune system is well established and supports their mutually affecting relationship. Many animal selection lines have been created according to individual behavioral or neuroendocrine responses to stress. Here we present two chicken lines selected for 25 generations for their primary antibody response to immunization with sheep red blood cells (SRBC), as well as the control line from the same parental strain. These selection lines show differential HPA axis reactivity next to the well documented differences in immunity. Here we report that re-sampling of blood in some of the birds caused significant effects on several immune parameters in a line-specific manner. We conclude that the difference in HPA axis reactivity is related to line-specific differential modulation of immune parameters due to mild stress and that technical procedures such as repeated blood sampling procedures may significantly alter results.

Introduction

The bidirectional relationship between the neuroendocrine system and the immune system, and the complexity of this relationship is still being unraveled, but is widely accepted (Beishuizen and Thijs, 2004). Genetic background and environmental factors determine the phenotype of the individual (Ellenbroek and Cools, 2002). Within this interplay there is a profound and complex relationship which is reflected in stress handling and disease resistance in the individual (Elenkov and Chrousos, 2006). The influence of genetic background on disease resistance is often investigated in animal model species using selection lines that are created based on different behavior in experimental settings and/or on different endocrine responses to stress (Lindqvist et al., 2007; Owen et al., 2008). The extremes of such selection lines have shown similarities in typical behavior and their endocrine responses. These extremes are characterized by either a hypo reactive HPA axis with active behavior (when given the possibility) or a hyper reactive HPA axis with less behavioral activity (often more freezing). This dichotomy has been found in many species, such as: rodents, chicken and fish (Koolhaas et al, 2007, Øverli et al, 2007).

Birds are known to be more stress sensitive than other species. For example, often technical procedures are done without anesthetic because anesthetizing can cause enough stress for the animal to die. Selection lines have been used before to investigate stress effects on the immune system (Ellenbroek and Cools, 2002). Here we present a selection line of layer hens that has been selected for 25 generations for their primary antibody (Ab) response to immunization with sheep red blood cells (SRBC). Two lines were divergently selected: the High (H) responders which produce high antibody levels to SRBC after immunization, the Low (L) responders which produce low antibody levels to SRBC after immunization. Next a random bred Control (C) line from the same parental stock (Parmentier et al., 1994) was maintained. Interestingly, almost all immune parameters of these lines differ, including various parameters of the innate and adaptive immune systems as well as humoral and cellular immune parameters (Parmentier et al, 1994, 1995, 1996, 2002, 2004; Kreukniet et al, 1996). Recently, we reported that these lines differ in HPA axis reactivity (Adriaansen-Tennekes et al, 2009a). The Low line birds have a hyper reactive HPA axis and the High line birds a hypo reactive HPA axis. Here we report that double blood drawing caused line-specific differences in several immune parameters.

Materials and Methods

Chickens, housing and feed

ISA Brown (Warren) medium heavy layer hens from three lines were used. The selection lines were selected for 25 generations prior to this experiment for either high or low primary antibody responses at 5 days after intramuscular immunization (without the use of adjuvant) with SRBC at the age of 35 days. The randomly bred third line is the control line, originating from the same parental stock. The experimental group consisted of 72 hens evenly divided over the three selection lines, but not immunized with SRBC.

The birds were kept according to routine procedures for layer hens in individual battery cages. The light regimen was 14 h of light (5.00 a.m. to 7.00 p.m.), and temperatures were between 16 and 21°C. The birds were fed *ad libitum* with a commercial diet (180 g/kg CP, 3460 kcal/kg) with free access to water. Birds were weighed weekly, prior to the sampling procedure, therefore making them accustomed to handling.

All experiments were approved by the Animal Welfare committee of Wageningen University.

Blood sampling and corticosterone determination

Blood samples were taken by wing venipuncture between 8.00 a.m. and 9.00 a.m., and the sampling procedure took a maximum of two minutes per bird. The selection line birds were all sampled at random so all lines were sampled evenly over the whole sampling period, while 18 birds were re-sampled. The birds were sampled at nine weeks of age, and the sampling procedure was considered to be comparable to a mild stressor (Webb and Mashaly, 1984). Blood (2 ml) was collected in a syringe and divided between a serum tube and a tube with heparin. Plasma and serum were collected after centrifugation of the samples and then stored at -20º C until further analysis. Plasma corticosterone was determined using a radioimmunoassay kit with detection interval 0 – 2000 ng/ml (IDS, Inc., Bolton, UK).

Humoral immune parameters

Specific antibodies directed at vaccines IBD and NCD were determined as described earlier (Ploegaert et al, 2007). Natural antibodies directed at LTA, LPS and KLH were determined as described earlier by Parmentier et al (2004). Complement activity (classical = CPW and alternative = APW) in serum was determined by the procedure described by Parmentier et al (2002).

Cellular immune parameters

Whole blood was diluted 1:30 and subsequently used for lymphocyte stimulation test (LST) with T- and B-cell mitogens, Con A and LPS according to the procedure described by Adriaansen-Tennekes et al (2009b *submitted*). Peripheral blood mononuclear cells (PBMC) were isolated using a Histopaque 1.077 gradient. Cells were washed, counted and diluted to a 10^7 cells/ml suspension. LST with PBMC was done according to the previously described procedure by Adriaansen-Tennekes et al (2009b *submitted*), with the T- and B-cell mitogens Con A and LPS as well as the RPMI control. All LSTs are presented in counts per minute. Nitrite production by monocytes was determined using PBMC, with and without (=control) LPS and according to the procedure described by Adriaansen-Tennekes et al (2009b *submitted*).

Statistical analysis

All data not normally distributed were omitted from analysis. The mean differences due to blood sampling and line were tested with Bonferroni's. Differences between the selection lines were determined by multiple comparison of the means. All data are presented as mean ± SEM. All analyses were according to SAS procedures (SAS, 2003).

Results and Discussion

Here we report the modulatory effects of a standard blood sampling procedure and repeated blood sampling, in two selection lines of chicken, together with the control line, on corticosterone levels and immune parameters.

As the High and Low line birds displayed differential HPA axis reactivity (Adriaansen et al., 2009a), we concentrated on these birds rather than birds from the Control line. All antibody levels (vaccine specific: IBD, NCD; natural antibodies: LTA, LPS and KLH) were not affected by the blood sampling procedure, but these parameters did reflect the line characteristics, with the High line birds showing the highest titers, followed by respectively the Control and Low lines. Repetition of blood sampling caused a consistent rise in corticosterone levels in all selection lines, with the same line difference as was seen after a single sampling procedure. The Low line birds showed the greatest rise in corticosterone.

The double blood drawing showed effects in several immune parameters such as the whole blood (WB) LST controls, WB LST with LPS, number of cells and APW wherein all these parameters were lower in the birds subjected to a second blood sample (Table 1). Although not statistically significant, the monocyte medium controls showed the same trend as the previous parameters. Only the PBMC LST controls were higher in the double sampled hens when compared with the PBMC LST controls of the birds sampled once. Interestingly, three of the five parameters which showed effects due to double sampling, showed significant moderate to strong correlations with the measured levels of corticosterone (WB LST controls: r= 0.50574, PBMC LST controls: r= 0.70236 and number of cells: r=0.52591).

The selection lines were affected differently by the double sampling (Table 1). The High line birds showed significant decreased proliferative activity of the WB LST controls and PBMC LST with Con A when sampled twice, and the number of PBMC cells dropped significantly when sampled twice. On the other hand the Low line birds showed significantly decreased APW and CPW activity as well as an increased proliferative response of the PBMC LST controls.

The line-specific effects of the High line birds are in line with results found by other groups. Kiank et al (2006) also showed a reduced number of lymphocytes in stressed mice. The reduced proliferation of the PBMC's to ConA after the double blood drawing is most likely the result of stress effects via the sympathetic nervous system of these birds as was reported by Edgar et al (2003). The line-specific effects found in our Low line birds are not as easily explained, although Maes et al (1997) showed that complement activity increased more due to stress

Table 1. Measured corticosterone levels (ng/ml) and immune parameters in High (H), Control (C) and Low (L) line hens after one or two times blood drawing (BS).

Line[2]	BS	B	WB Control	WB LPS	WB Con A	PBMC Control	PBMC LPS	PBMC Con A	Cell N	Mono Control	Mono LPS	APW	CPW
H (n= 15)	1x	$6.9^{a,b}$	748^{x}	2356	13792	271^{x}	297	$3448^{a,x}$	20.1^{x}	0.37	10.9	78.9^{b}	161.8^{b}
C (n= 21)		4.8^{b}	498	2023	15019	291	251	1254^{b}	25.8	0.09	7.2	70.8^{b}	$178.5^{a,b}$
L (n= 18)		12.4^{a}	573	2204	16674	222^{x}	204	1183^{b}	22.8	0.43	5.3	$164.0^{a,x}$	$196.4^{a,x}$
H (n= 9)	2x	$11.6^{a,b}$	375^{y}	1025	17605	307^{y}	331	2101^{y}	10.2^{y}	0.02	15.7	48.8^{b}	146.8
C (n= 4)		6.4^{b}	356	1259	17976	-	-	-	-	0.00	-	63.0^{b}	177.0
L (n= 5)		20.1^{a}	445	1186	12364	406^{y}	409	2297	17.6	0.00	5.6	$112.2^{a,y}$	152.6^{y}
SEM		3.3	156	675	4543	54.4	99.3	520	4.83	0.21	6.1	18.1	15.5
Main Effects[2]													
Sampling		0.1079 1>2	NS	0.0816 1>2	NS	0.0773 2>1	NS	NS	0.0035 1>2	NS	NS	0.0575 1>2	NS
Line		0.0144 L>H>C	NS	NS	NS	NS	NS	0.0252 H>L>C	NS	NS	NS	0.0002 L>C>H	NS
SxL		NS	NS	NS	NS	NS	NS	0.0408	NS	NS	NS	NS	NS

*BS = blood sampling being either 1x (once) or 2x (repeated); B = corticosterone levels; WB control = LST of whole blood controls; WB LPS = LST of whole blood with B-cell mitogen LPS; PBMC control = LST of PBMC controls; PBMC Con A = LST of PBMC with T-cell mitogen Con A; Cell N = number of cells/ml; APW = alternative complement activity; CPW = classical complement activity.

[1]Data are group (least square) mean. Number of birds per group are noted in the first column as n = X. Within blood sampling contrast between lines with no common superscript differ significantly: a,b< 0.05. Statistical difference within line between sampling (one or two): x,y< 0.05.

[2]Main effects based on Main Effect Means: Sampling = effect due to one or two blood samples; Line= line effect; SxL = sampling by line effect. NS = no significance, otherwise p value is noted

in students with high stress perception. De Groot et al (2000) showed that WB and PBMC LST showed different effects due to housing in pigs. Leandro et al (2006) also report that stress effects depend on the compartment of the immune system investigated, as for example reversed effects were found in cells from spleen and thymus. Different stressors have been shown to evoke different stress responses (Bowers et al, 2008). This, together with the line-specific differences in responses to stressors, as well as the many different immune parameters used in such studies requires further in-depth studies to reveal the underlying mechanisms and their possible relationships.

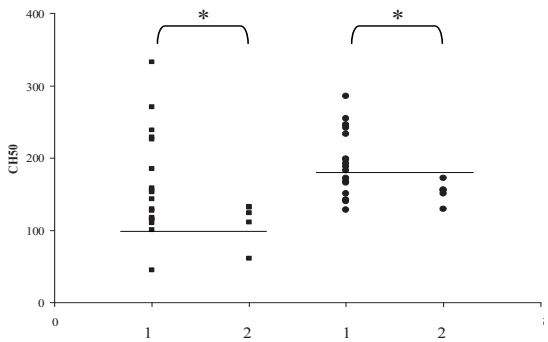

Figure 1. Scatter plot of the measured APW (■) and CPW (●) activity in Low line birds sampled either once (n = 18) or twice (n = 5). Statistical difference within line between sampling: * $p<0.05$. The line represents the average of all birds: APW = 95 and CPW = 175 CH50.

As the birds were accustomed to weekly handling, the sampling procedure was considered to be comparable to a mild stressor. Mild stress only elevates corticosterone levels slightly (Hazard et al, 2008; Salomé et al, 2006). Although no correlation with LPS was found, Palacios et al (2007) found a relationship between corticosterone levels and WB LST with LPS in free living birds. Most of the found effects due to double blood drawing, but that were not line-specific, showed correlations with the measured corticosterone levels, making it likely that these effects were the result of the rise in corticosterone.

In conclusion, our results corroborate that chicken are very stress sensitive and that repetition of a sampling procedure may skew immunological results and will do this in a line-specific manner in selection lines based on differential HPA axis reactivity. This may especially affect cellular immune parameters and APW of complement. Furthermore, as these selection lines have already been extensively investigated for various immune traits, they present a suitable model for investigating stress reactivity and disease resistance with the underlying mechanisms.

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CHAPTER

V

Chicken lines divergently selected for Antibody response to SRBC show line-specific differences in sensitivity to immunomodulation by diet, part I: humoral parameters

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Poultry Science (2009) 88; 1869-1878

Abstract

Individual differences in nutrient sensitivity have been suggested to be related with differences in stress sensitivity. Here we used layer hens divergently selected for high and low specific antibody responses to SRBC (i.e., low line hens and high line hens), reflecting a genetically based differential immune competence. The parental line of these hens was randomly bred as the control line and was used as well. Recently, we showed that these selection lines differ in their stress reactivity; the low line birds show a higher hypothalamic-pituitary-adrenal (HPA) axis reactivity. To examine maternal effects and neonatal nutritional exposure on nutrient sensitivity, we studied 2 subsequent generations. This also created the opportunity to examine egg production in these birds. The 3 lines were fed 2 different nutritionally complete layer feeds for a period of 22 wk in the first generation. The second generation was fed from hatch with the experimental diets. At several time intervals, parameters reflecting humoral immunity were determined such as specific antibody to Newcastle disease and infectious bursal disease vaccines; levels of natural antibodies binding lipopolysaccharide, lipoteichoic acid, and keyhole limpet hemocyanin; and classical and alternative complement activity. The most pronounced dietary-induced effects were found in the low line birds of the first generation: specific antibody titers to Newcastle disease vaccine were significantly elevated by 1 of the 2 diets. In the second generation, significant differences were found in lipoteichoic acid natural antibodies of the control and low line hens. At the end of the observation period of egg parameters, a significant difference in egg weight was found in birds of the high line. Our results suggest that nutritional differences have immunomodulatory effects on innate and adaptive humoral immune parameters in birds with high HPA axis reactivity and affect egg production in birds with low HPA axis reactivity**.**

Introduction

Diet is the main source of nutrients and energy necessary for optimal physiological responsiveness, maintaining health, survival, and eventually reproduction. Nutritional and metabolic exposure of fetus and neonate may have long-term programming effects into adulthood on, for instance, immune competence. Traditionally, immune competence is defined as the levels of (natural or specific) Ab or complement activity without immune challenge (Star et al., 2007); the higher the levels, the greater the immune competence. Also in adults, dietary modulatory effects, both positive as well as negative, on immune responses are known. Nutritional immunomodulation is thought to contribute to survival of the offspring in ever-changing environmental conditions (Burdge et al., 2007).

To date, several groups have reported the occurrence of individual differences in sensitivity for immunomodulation by diet (Grimble, 2001; Desiere, 2004). Albers et al. (2005) proposed immune parameters that may be useful to measure immunomodulation by diet in human nutritional studies. Hamer et al. (2004) suggested that individual differences should be investigated in animal stress models. Recently, we showed that genetic selection chicken lines differ in stress reactivity (Adriaansen-Tennekes et al., 2009), next to their well-documented difference in antibody (Ab) production (Parmentier et al., 1994, 1996, 1998a, 2004). The birds selected for high Ab responses to SRBC (high line) have higher levels of (natural) Ab and also show higher Ab production to various other antigens after immunization. Furthermore, these lines differ in almost every other aspect of innate and specific humoral and cellular immunity (Parmentier et al., 1994, 1995, 1996, 2002, 2004; Kreukniet et al., 1996), making these birds a suitable model to study the mechanisms underlying the differences in sensitivity for immunomodulation by diet.

To investigate individual differences in sensitivity to immunomodulation by diet, 2 different layer diets, both nutritionally complete, were used, whereas parameters of innate and specific humoral immunity were investigated in 2 subsequent generations of layer hens. Because these selection lines differ in stress reactivity, effects due to diet may differ in the these lines due to the different neuroendocrine regulation in these birds; therefore, egg production parameters were determined as well in the first generation. To establish a possible role of the hypothalamic-pituitary-adrenal (HPA) axis, corticosterone was measured. Birds originated from the 25th generation of ISA Warren brown medium heavy layers that were divergently selected in the past for high or low Ab responses to SRBC at 5 d after immunization with SRBC at 5 wk of age. A randombred control line from the same parental line was held throughout these generations as well.

Studying 2 subsequent generations provided the opportunity to study the consequence of maternal and neonatal nutritional exposure to 2 different diets on humoral immune parameters of chicks from the following generation. Until the age of 11 wk, the first generation of birds was supplied with normal commercially obtained starter-grower diets, after which they were supplied with either 1 of 2 experimental diets, differing slightly in protein levels. Egg production parameters were recorded (number and quality of eggs, as well as egg weight). Eggs from the first generation were used to form the second generation that was fed the same diet as the corresponding hen from hatch until the end of the experiment (8 wk of age). During a period of 23 wk for the first and 1 wk for the second generation, parameters of innate and adaptive immunity were measured in these birds. Here we report that minor dietary differences selectively affected humoral components of the chicken immune system and that these selection lines show differences in sensitivity to immunomodulation by diet, with a role for the HPA axis. Effects of the diets on cellular immunity will be shown in a following paper.

Materials and Methods

Birds

Two generations of ISA Brown (Warren) medium heavy layer hens from 3 lines were used. The lines were selected for 25 generations before this experiment for either high or low primary Ab responses at 5 d after i.m. immunization (without the use of adjuvant) with SRBC at the age of 35 d. The randomly bred third line is the control line, originating from the same parental stock. The experimental first generation consisted of 72 hens evenly divided over the 3 selection lines, but not immunized with SRBC. In the second offspring generation, 150 chicks, 50 of each line, were used, again not immunized with SRBC.

All experiments were approved by the Animal Welfare Committee of Wageningen University.

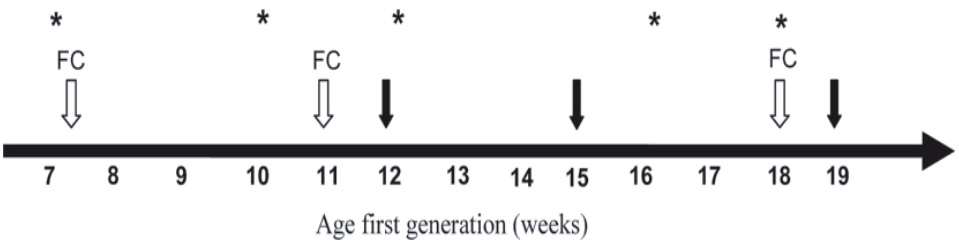

Figure 1. Timeline for the experimental design of the blood sampling for the first generation of hens. At 33 wk of age, the first generation was sampled as well; this is not depicted in the figure. Solid arrows represent blood sampling times, and open arrows represent a feed change (FC). An asterisk (*) represents regular vaccinations.

Experimental Design

The first generation of birds was kept according to routine procedures for layer hens in individual battery cages. The second generation hens were kept at 6 (2 birds of each line per cage) birds per enriched cage (2.28 m^2) in a floor housing system. For both generations, the light regimen was 14 h of light (0500 to 1900 h) and temperatures were between 16 and 21°C. At hatch, all chicks of the first generation were vaccinated against Marek's disease (Poulvac, Fort Dodge Animal Health, Vaals, the Netherlands) and infectious bronchitis (IB, MA 5, Nobilis-Intervet, Boxmeer, the Netherlands), IB at d 71 (primer, Poulvac) and d 126 (H52, Nobilis), infectious bursal disease (IBD, Gumboro, D78, Nobilis) at d 22, infectious laryngotracheitis (Nobilis) at d 86, and Newcastle disease (NCD, clone 30, Nobilis) at d 10, 42, 51, and 114 of age. The second generation birds received

vaccinations against Marek's disease and IB at hatch, IBD at d 21, and NCD at d 10, 28, and 40 of age.

The first generation was sampled by wing venipuncture according to the schedule as depicted in Figure 1. The second generation was sampled once at 8 wk of age, 1 wk after the change from starter to grower feed, also by wing venipuncture. Blood was collected in a syringe and thereafter divided over a serum tube and heparinized tube. After centrifugation, serum and plasma were collected and stored at −20°C until further analysis. Plasma corticosterone was determined using a RIA kit (IDS Inc., Bolton, UK).

Experimental Feed

All birds received diets that were appropriate for their age, being: starter (from hatch to 6 wk of age), grower (7 to 17 wk), and layer diet (from 18 wk on). For each appropriate age, 2 identical experimental diets were made only differing in the cultivation method of the raw ingredients [wheat, barley, triticale, peas, maize (*Zea mays*), and soy]. Each raw ingredient (e.g., wheat A) was used for all the produced experimental diets designated diet A. Diet composition and macronutrient contents are shown in Tables 1 and 2, respectively. The main difference between diets A and B was protein content (B > A). The first generation of birds received commercial diets from hatch, a conventional diet from 7.5 wk of age [specially made because the experimental diets were of a different structure (less coarse) than the commercial diet] and the experimental diets from the age of 11 wk. The second generation received the experimental diets from hatch to the end of the observation period.

Reagents

Escherichia coli-derived lipopolysaccharide (LPS) and *Staphylococcus aureus*-derived lipoteichoic acid (LTA) were from Sigma Chemical Co. (St. Louis, MO). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA).

Specific and Natural Ab

Levels of natural and specific Ab (NAb and SpAb, respectively) were determined by indirect 2-step ELISA (Parmentier et al., 2004). In short, 96-well medium binding plates were coated overnight at 4°C with LPS, LTA, KLH, NCD, or IBD. The next day, 2-fold diluted plasma was added and incubated for 1 h at room temperature. Detection of binding of Ab to antigen was performed with horseradish peroxidase labeled rabbit antichicken IgG_{H+L} conjugate (RACh/IgG_{H+L}/PO, Nordic, Tilburg, the Netherlands) and the substrate tetramethylbenzidine and

stopped with H_2O_2. Extinction was measured at 450 nm (Multiscan, Labsystems, Helsinki, Finland). The titers were expressed as the log2 values of the highest dilution giving a positive reaction. Because small effects are expected due to diet, delta titers were calculated by increase-decrease in titer between 2 subsequent measurements to determine immune responsiveness in the first generation (e.g., titer at wk 15 – wk 12, titer at wk 33 – wk 12).

Table 1. Feed composition per age group[1]

Age group	Starter 0 – 6 weeks	Grower 7 – 17 weeks	Layer from 18 weeks
Raw ingredients			
Maize	20.05	20.06	25.01
Wheat	30.08	26.51	25.23
Barley	5.01	10.03	5.00
Triticale	12.08	0.00	0.00
Soybeans (heated)	0.00	10.21	19.87
Soy flakes	10.18	20.06	0.00
Peas	10.03	10.03	10.00
Supplements			
Potato protein	7.02	0.00	2.50
MonoCalcFos	1.13	0.73	1.01
FX layers premix	1.00	1.00	1.00
Fat (plant origin)	1.50	0.00	0.52
Salt	0.07	0.09	0.06
Chalk	1.65	1.16	7.65
Shells (broken)	0.00	0.00	2.00
$NaCO_3$	0.09	0.08	0.00
Methionine	0.11	0.04	0.15

[1]All numbers reflect the percentage of the ingredient in the total feed. The raw ingredients for diets A and B were from different sources, and all supplements were from the same bulk source.

Hemolytic Complement Assay

Serum from all birds was diluted serially in 96-well flat-bottom plates and incubated with either sensitized bovine or sheep erythrocytes to measure alternative (APW) or classical complement pathway (CPW), respectively. Every 30 min of the 1.5-h incubation period, the plates were shaken. Lysis (the amount of light scattering by erythrocytes) was measured by measuring at the wavelength of 690 nm. The extinctions were transformed by a log-log equation (Von Krogh, 1916), and the hemolytic titer was expressed as the titer with 50% lysis of red blood cells (CH50 U/mL).

Egg Collection

Eggs were collected each day from the start of the laying period (on average at the age of 20 wk) up to 5 mo thereafter. The following egg types were classified as low-quality eggs: textured egg, double yolk, pimpled egg, thin shell, mini eggs. Data analysis was performed on the monthly data of 5 consecutive months. Hens

that laid less than 50 eggs in the whole observation period were omitted from analysis.

Two months after the start of egg laying, 4 wk (age in weeks: 25, 27, 28, and 29) eggs were collected and weighed.

Table 2. Macronutrient content in the experimental feeds[1]

	First Generation				Second Generation			
	Grower		Layer		Starter		Grower	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Energy KJ/kg	14480	14610	14487	14302	14729	14882	15231	15102
Ash content g/kg	51	48	113	114	64	51	55	54
Raw fibre g/kg	43	42	36	40	36	34	39	39
Tot. carbohydrates g/kg	546	539	555	538	624	620	585	574
Crude fat g/kg	61	59	69	64	42	42	62	53
Protein g/kg	173	192	147	164	151	164	176	199
Moisture g/kg	126	120	116	120	119	123	122	120
Chloride g/kg	1.7	2.0	1.3	1.4	2.3	2.1	1.9	2.3

[1]Starter feed was given 0 to 6 wk of age, grower feed from 7 to 17 wk of age, and layer from 18 wk of age on. For all of the A diets, the same batches of raw ingredients were used, as was done for all the B diets.

Statistical Analysis

The differences in all parameters were analyzed by a 3-way ANOVA for the effect of diet, line, time, and their interactions using the repeated measurement procedure (with a bird nested within diet and line option). The mean differences due to diet and line were tested with Bonferroni's correction. Differences between the selection lines were determined by multiple comparisons of the means. All data are presented as mean ± SEM. All analyses were according to SAS procedures (SAS Institute, 2003).

Results

First Generation

Corticosterone.

The measured corticosterone levels of the first generation are depicted in Figure 2. One bird was omitted from data analysis because it was an extreme outlier (low line bird fed diet A, response at wk 11 was 1915.2 ng/mL). A week after the change to the experimental diets, a significant diet effect was found, wherein all birds fed diet A showed higher corticosterone levels than the birds fed diet B [main effect means (MEM) were, respectively, 17.3 and 8.0 ng/mL, P = 0.0114]. This difference was significant in the low line birds, wherein the birds fed diet A showed an increase in the corticosterone levels when compared with the low line birds fed diet B. The corticosterone levels of all hens showed a significant increase in time. The low line birds fed diet A showed higher corticosterone levels over time, starting at 15 wk of age, when compared with the low line birds fed diet B (repeated measures: P = 0.0262).

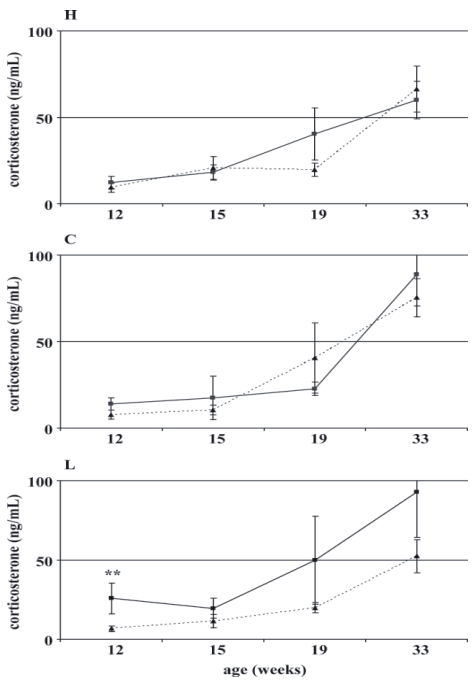

Figure 2. Corticosterone levels during experimental period of the first generation of hens. High (H), control (C), and low (L) line birds on feed A (■, solid line) or feed B (▲, hatched line). Data are mean ± SEM. In the first generation, n = 11 to 13 birds/line per feed. Statistical difference within line between diets: **P < 0.01.

SpAb to NCD and IBD Vaccine Strains.

The SpAb levels to the vaccine strain NCD showed a significant diet × line interaction (P = 0.0140, Table 3), wherein the low line birds fed diet A showed significantly higher SpAb levels over time when compared with their counterparts fed diet B (P = 0.0027). The high line birds showed the reverse, wherein the diet B-fed birds showed a consistently higher increase (Figure 3). Within the delta titers, again the significant diet × line interaction was seen (P = 0.0011). The characteristic line difference in response was only found in the diet B-fed birds (within diet contrast between lines). Within the diet A-fed hens, the low line hens showed an increased SpAb production to NCD during the first weeks (Figure 3).

The absolute SpAb levels to the vaccine strain IBD showed a significant interaction between line and diet over time (Table 3), reflecting differences in kinetics of the responses due to the experimental diets (diet × line × time: P = 0.0272). Interestingly, the low line birds fed diet A showed the highest absolute levels over time, when compared with all other groups. The SpAb response (delta titers) of anti-IBD SpAb was reversed when regarding the selection line characteristics (Figure 3), wherein the low line birds showed the least decrease in SpAb over time (Table 4); this effect was greatest in the diet B-fed birds.

NAb.

The KLH binding Ab levels of the birds are considered to be part of the pool of NAb because these birds were not exposed to KLH throughout the experiment. The absolute KLH titers reflected the line characteristics, wherein the diet B-fed birds showed the greater contrasts between the lines. The delta KLH binding NAb showed a moderate interaction between diet and line (P = 0.074, Table 3), in which diet B showed the characteristic line effect wherein the high line birds showed the greatest increase in titers over time. The high and control line birds fed diet B consistently showed a greater KLH binding NAb increase (delta titer) after the switch to the experimental diets when compared with the same line of birds fed diet A. The low line birds fed diet A showed consistently higher KLH binding NAb after the diet change when compared with the low line birds fed diet B.

The absolute levels of NAb binding LTA were higher in the high line than in the other lines (Table 3), in which diet A showed greater contrasts between the lines than diet B. The increases in titers over time were not different (delta titers), which is in contrast with the characteristics of these lines.

The line characteristics were reversed in the absolute LPS binding NAb levels (Table 3, P = 0.0081). Diet B showed the greatest contrasts between the selection lines when compared with diet A. The absolute LPS binding NAb levels

Table 3. Specific and natural (absolute and delta) plasma antibody titers[1] during 22 wk (first generation) of feeding with the experimental diets in the high (H), control (C), and low (L) line hens[2]

		Absolute titers					Delta titers				
Line	Diet	SpAb		NAb			SpAb		NAb		
		NCD	IBD	LTA	LPS	KLH	NCD	IBD	LTA	LPS	KLH
H	A	4.36^{a,b}	6.69	5.45^{a}	6.62^{b}	5.32^{a}	2.20^{a,b,x}	-1.62^{b}	1.35	2.17	1.64^{a,x}
C		3.79^{b}	6.94	4.49^{b}	6.91^{a,b}	4.80^{a,b}	2.11^{b}	-0.79^{a,b}	1.21	1.86	1.72^{a}
L		4.70^{a,x}	7.02	3.83^{b}	7.55^{a,x}	4.28^{b}	3.02^{a,x}	-0.19^{a}	1.33	2.04	2.00^{a}
H	B	4.73^{a}	6.39	6.06^{a}	5.96^{b}	5.49^{a}	3.15^{a,x}	-1.54^{b}	1.50	1.61	2.24^{a,x}
C		3.88^{b}	6.26	4.51^{b}	7.12^{a}	5.09^{a}	2.29^{b}	-1.21^{b}	0.74	1.26	2.06^{a,b}
L		3.26^{b,x}	6.29	4.14^{b}	6.69^{a,x}	4.03^{b}	1.32^{c,x}	0.41^{a}	1.33	1.88	1.55^{b}
SEM		0.32	0.56	0.37	0.28	0.24	0.35	0.37	0.36	0.37	0.23
Main Effects[2]											
D		NS	NS	NS	0.0609 A>B	NS	NS	NS	NS	NS	NS
L		0.0701 H>L>C	NS	<0.0001 H>C>L	0.0081 L>C>H	<0.0001 H>C>L	NS	<0.0001 L>C>H	NS	NS	NS
DxL		0.0140	NS	NS	NS	NS	0.0011	NS	NS	NS	0.074
T		<0.0001	0.0006	<0.0001	<0.0001	<0.0001	<0.0001	0.0005	<0.0001	<0.0001	<0.0001
DxT		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
LxT		<0.0001	NS	NS	0.0545	NS	<0.0001	NS	NS	0.054	NS
DxLxT		NS	0.0272	NS	NS	NS	NS	0.024	NS	NS	NS

[a–c]Within-diet contrasts between lines with no common superscript differ significantly ($P < 0.05$).

[x]Statistical difference within line between diets ($P < 0.05$).

[1]Least squares means ± SEM of the complete observation period after analysis by a repeated measurement procedure after the change to the experimental diets. Titers are log2 of the reciprocal of the antibody dilution. The delta titer is increase-decrease in absolute titer between 2 subsequent measurements (e.g., wk 15 − wk 12). SpAb = specific antibody; NAb = natural antibody; NCD = Newcastle disease; IBD = infectious bursal disease; LTA = lipoteichoic acid; LPS = lipopolysaccharide; KLH = keyhole limpet hemocyanin.

[2]Data are group mean: first generation n = 11 to 13 birds/line per feed.

[3]D = diet; L = line; D × L = diet × line interaction; T = time; L × T = line × time interaction; D × T = diet × time interaction; D × L × T = diet × line × time interaction; NS = no significance, otherwise P-value is noted.

were marginally higher in all diet A-fed birds (P = 0.0609, Table 3). No differences due to line or diet were found in the delta titers of NAb binding LPS due to the change to the experimental diet (Table 3) when regarded over time. When individual time points were analyzed, both 3 wk after the change from conventional grower to experimental grower diet (wk 15), as well as the change from experimental grower to experimental layer diet (wk 23), significantly higher levels of NAb binding LPS (P = 0.0303 and P = 0.0141, respectively) were found in the diet A-fed birds irrespective of line (data not shown).

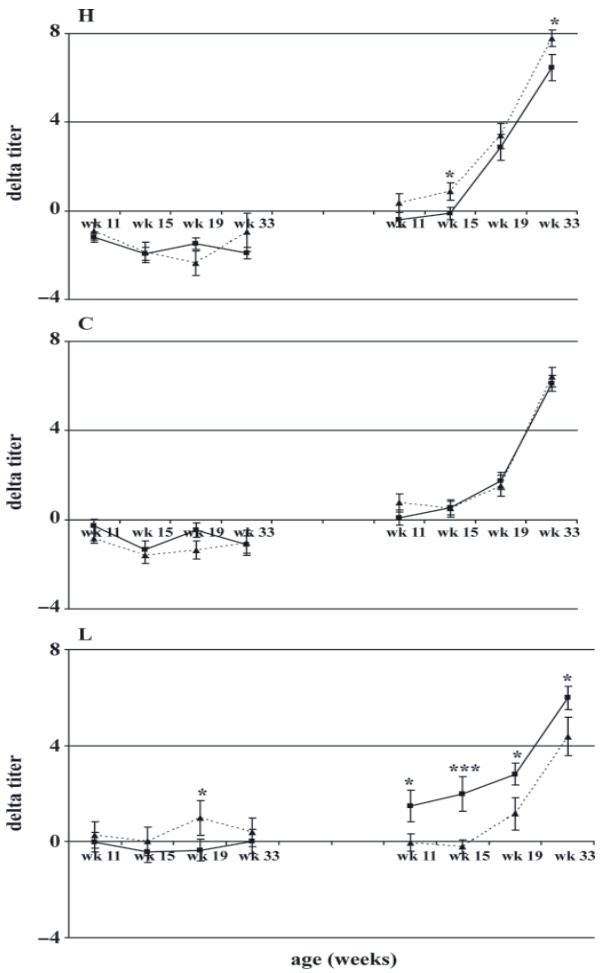

Figure 3. Delta titers for, respectively, infectious bursal disease and Newcastle disease specific antibodies during the observation period in the first generation of hens. High (H), control (C), and low (L) line birds on feed A (■, solid line) or feed B (▲, hatched line). Data are mean ± SEM. In the first generation, n = 11 to 13 birds/line per feed. Statistical difference within line between diets: *P < 0.05; ***P < 0.001.

Complement.

No significant diet effects were found in time. The APW was higher in low line birds than in control and high line birds (data not shown). For CPW levels, a significant interaction between line and time was found, but no significant line or diet effects were obtained (data not shown).

Correlations Between Corticosterone Levels and Immune Parameters.

No correlations with corticosterone were found at the first measurement of SpAb and NAb at 12 wk. At 15 wk, only a few correlations were found in the high and low line birds (data not shown). High line birds fed diet B showed a positive correlation between LPS NAb and corticosterone ($r = 0.62237$). The low line birds fed diet A showed a positive correlation between LTA NAb and corticosterone ($r = 0.58361$). At 19 wk, the amount of correlations increased, with no consistent correlations between the 2 time points (data not shown). The IBD vaccine SpAb of the control and low line hens were negatively correlated with the corresponding corticosterone levels ($r = -0.64687$ and $r = -0.80877$, respectively). The CPW was positively correlated with corticosterone in the high line birds fed diet A and the low line birds fed diet B ($r = 0.64029$ and $r = 0.76928$, respectively). The control line hens showed contrasting (A negative and B positive) correlations between the 2 diets between NCD titers and corticosterone. The control line birds fed diet B showed the most correlations between the immune parameters and corticosterone on the whole.

Egg Production Parameters.

The data from 4 hens were omitted from data analysis because they had produced less than 50 eggs during the whole observation period (1 high line hen fed diet A, 2 high line hens fed diet B, and 1 low line hen fed diet B).

There was a significant difference in the start of egg laying between the selection lines ($P = 0.0027$). The control line hens produced on average 12.8 eggs in the first month of laying, whereas the high and low line birds produced, respectively, 7.7 and 7.5 eggs on average. After the 5-mo collection period of eggs, a significant difference was found in the MEM of total number of eggs produced during the observation period. The MEM for the control line hens was 115.0 with, respectively, the high line and low line MEM being 109.6 and 106.9. The MEM for the control and low line birds were significantly different ($P = 0.0391$). No interactions were found due to the 2 diets.

The egg weights showed significant line differences in the first 3 wk ($P = 0.0003$, $P = 0.0292$, and $P = 0.0039$, respectively), with the control line birds having the heaviest eggs followed, respectively, by the low and high line birds

(e.g., MEM line at wk 29: 46.0 – 52.5 – 50.8 g, respectively, the high – control – low line). At wk 29, all hens fed diet B consistently had heavier eggs than the diet A-fed birds. This difference was significant for the high line birds (42.4 g diet A, 49.6 g diet B; P = 0.0492).

Table 4. Antibody titers[1] directed to Newcastle disease (NCD) and infectious bursal disease (IBD) vaccines as well as binding to lipoteichoic acid (LTA), lipopolysaccharide (LPS), and keyhole limpet hemocyanin (KLH) at 8 wk of age in the second generation of feeding with the experimental diets from hatch in the high (H), control (C), and low (L) line hens[2]

		SpAb		NAb		
Line	Diet	NCD	IBD	KLH	LTA	LPS
H	A	1.58^{a}	5.27^{a}	0.68^{a}	7.27^{a}	1.94^{a}
C		1.62^{a}	2.98^{b}	0.60^{a}	7.91^{a,x}	1.58^{a}
L		0.79^{b}	1.23^{c}	0.07^{b}	6.01^{b,x}	0.80^{b}
H	B	1.40^{a}	5.51^{a}	0.58^{a}	7.24^{a}	1.53^{a}
C		1.59^{a}	3.59^{b}	0.50^{a}	6.42^{a,x}	1.73^{a}
L		0.83^{b}	0.88^{c}	-0.05^{b}	4.88^{b,x}	0.62^{b}
SEM		0.14	0.59	0.09	0.36	0.21
Main effects[2]						
Diet		NS	NS	NS	0.0034 A>B	NS
Line		<0.0001 C>H>L	<0.0001 H>C>L	<0.0001 H>C>L	<0.0001 H>C>L	<0.0001 H>C>L
DxL		NS	NS	NS	NS	NS

[a-c] Means within line between diets with no common superscript differ significantly (P < 0.05).
[x] Within-line contrasts between diets with common superscript differ significantly (P < 0.05).
[1] Least squares means ± SEM of the complete observation period after analysis by a repeated measurement procedure after the change to the experimental diets. Titers are log2 of the reciprocal of the antibody dilution. SpAb = specific antibody; NAb = natural antibody.
[2] Data are group mean: second generation n = 21 to 26 birds/line per feed.
[3] D = diet; L = line; D × L = diet × line interaction; NS = no significance, otherwise P-value is noted.

Second Generation

Corticosterone.

In the second generation of hens, a significant difference in corticosterone levels was found (data not shown). The birds of the low line had significantly higher corticosterone levels when compared with the control (P = 0.0457) and high line birds (P = 0.0437). No discernable effects due to the 2 diets were detected.

SpAb to NCD and IBD Vaccine Strains.

In the second generation, the anti-NCD SpAb levels almost reflected the expected immune competence of the selection lines (P < 0.0001, Table 4). The

highest levels of Ab binding NCD were found, respectively, in the control and high line.

When regarding the anti-IBD SpAb levels, again the line characteristics were confirmed and no effects were found due to the experimental diets ($P < 0.0001$, Table 4).

NAb.

In the second generation, the KLH binding NAb levels at the sampling moment (i.e., 1 wk after the diet change) reflected the characteristics of the selection lines ($P < 0.0001$, Table 4).

The NAb binding to LTA showed a significant diet effect, with the birds fed diet A showing greater levels of NAb binding LTA than the diet B-fed birds ($P = 0.0034$). This effect was greatest in the control line and low line birds. The high line hens showed no difference due to diet at this moment.

There were no differences in the LPS binding NAb levels a week after the birds changed from starter to grower diet (Table 4); therefore, no effect on the immune competence was found for this parameter.

Complement.

In the second generation, no effects due to diet were found in APW or the CPW levels (data not shown). CPW activity was comparable in all lines, while APW showed a significant line effect ($P = 0.0106$), wherein the low line birds showed significantly lower activity than the high and control line birds.

Table 5. Pearson correlations (r) between corticosterone levels and titers to Newcastle disease (NCD) and infectious bursal disease (IBD) as well as lipoteichoic acid (LTA), lipopolysaccharide (LPS), and keyhole limpet hemocyanin (KLH) and complement [alternative complement pathway (APW) and classical complement pathway (CPW)] at 8 wk of age in the second generation of feeding with the experimental diets from hatch in the high (H), control (C), and low (L) line hens[1]

		Second Generation						
Line	Diet	NCD	IBD	KLH	LTA	LPS	APW	CPW
H	A	-	-	-	-	-0.42185 0.0450	-	-
C		-	0.43149 0.0398	-	-	0.46410 0.0257	-	-
L		-	-	0.45501 0.0291	-	0.53927 0.0079	0.35604 0.0954	0.39981 0.0587
H	B	-	-	-0.44901 0.0316	-	-	-	-
C		-	-	-	-	-	-	-
L		-	-	-	-	-	-	-

[1] Data are presented with r-value and P-value.

Correlations Between Corticosterone Levels and Immune Parameters.

In the low line birds fed diet A, several positive correlations with corticosterone were found (with KLH, LPS, APW, and CPW; Table 5). Control line hens fed diet A showed a positive correlation between IBD and LPS with corticosterone. In the high line birds, only negative correlations with corticosterone were found. Interestingly, all lines fed diet A showed a correlation with LPS titers, although the correlation was negative for the high line birds and positive for the control and low line birds.

Discussion

We studied the sensitivity of 3 chicken lines that had been divergently selected in the past for their specific primary Ab response to the T-cell-dependent antigen SRBC, to immunomodulation by 2 nutritionally complete but different diets. In addition, feeding these diets to birds of a first generation that were grown up with conventional diet next to birds of a second generation that were fed the experimental diets from hatch was studied. The latter part was performed to establish possible maternal or neonatal, or both, nutritional exposure. Corticosterone levels were measured to establish potential stress-related effects due to feed and feed changes. Because these lines show different neuroendocrine regulation, differential effects due to diet were considered possible; therefore, egg production parameters were established as well.

The higher corticosterone levels in all the birds fed diet A suggest a reaction of the HPA axis to dietary component(s) in diet A. The HPA axis responses to dietary factors such as protein in diets, LPS, and β-glucans have been shown previously (Andersson et al., 2000; Benedict et al., 2005; Breivik et al., 2005; Shini et al., 2008). It is likely that a corticosterone response was exhibited by the high line birds fed diet A, but due to our sampling schedule (a week after the change to the experimental diets), we did not measure this because the high line birds are characterized by a hyporeactive HPA axis, with a decreased and more rapid response (Adriaansen-Tennekes et al., 2009). The change from the experimental grower to experimental layer feeds at the age of 18 wk showed a small but consistent divergence of the corticosterone levels a week later, which in the low line birds progressed significantly over time. This may represent a form of chronic stress to the dietary component(s) that have caused the HPA axis response, as is well known with chronic mild stress, and the accompanying mild elevation of corticosterone levels (El-Lethey et al., 2003; Silberman et al., 2004).

Overall, the absolute titers reflected the line characteristics in our experiment with the exception of the LPS NAb, which has been seen before (Parmentier et al., 1998b). Interestingly, the effects of the diets are reflected mainly in the increase-decrease production of Ab, expressed here as the delta titers. Diet B, on the other hand, showed the line characteristics consistently in the absolute titers as well as the level of production of Ab (delta titers) after the change to the experimental diets. Antibody titers have been shown to have a strong genetic component that is not easily modified (Råberg et al., 2003).

The low line birds showed a corticosterone response due to diet A and subsequently an increase and less decrease in, respectively, the NCD and IBD

titers. This was only seen in the vaccine titers and not the NAb, which may be due to the same mechanisms as was seen in stress-susceptible Ab and stress-unsusceptible Ab before (El-Lethey et al., 2003). Another possible mechanism may be the presence of LPS or β-glucans, or both, in the diets, which may have acted as immunological adjuvants to the vaccines. Immunomodulation due to wheat has been attributed to LPS contamination before (Yamazaki et al., 2008), as well as β-glucans from barley or molds, or both (Akramienė et al., 2007). Kreukniet et al. (1992) showed earlier that the line characteristics of these selection lines disappeared when immunizations were done in the presence of adjuvant such as complete Freund's adjuvant. Whether our findings also rest on an adjuvant's like effect of a dietary component (O'Hagan et al., 2001) remains to be established.

It is assumed that when the immune system is challenged, this is at the cost of production parameters such as BW gain and egg production. Indeed, in previous work, the same selection lines were found to differ in BW gain and egg production (Parmentier et al., 1996). In the current experiment, a significant difference in egg production was found in these lines, with the control line birds starting laying earlier than the high and low line birds. This is most likely the subsequent effect of the selection criteria. The weight of eggs from control line birds was always greatest and the high line birds always produced eggs with the lowest weights, with the eggs from the low line birds in between. Egg weight progresses with age of the hen and is most likely the main reason that the control line birds produced heavier eggs. Because the high and low line birds start egg laying at approximately the same time, the difference in egg weight seems related to the level of Ab production. Birds from the low line with low Ab production produce heavier eggs than birds of the high line, with high Ab production. At the end of the observation period, the eggs produced by the high line birds fed diet B were significantly heavier than the eggs produced by the high line birds fed diet A. This may indicate that the production parameters of the high line birds were affected by diet instead of humoral immune parameters.

In the second generation, no differences in corticosterone levels were found between the diets, but this may be the result of epigenetic effects (Macrì and Würbel, 2006) or because these birds received these diets from hatch, or both. In this generation, all SpAb as well as NAb levels reflected the line characteristics. Interestingly, the LPS NAb were line consistent in this generation, contrary to what was found in the first generation and previously (Parmentier et al., 1998b). Only the LTA NAb levels reflected a diet effect in the control and low line birds of this generation. Again, a decrease in line contrasts was found in the diet A-fed birds. Although no differences in corticosterone levels were measured, the most

consistent correlations with corticosterone were found in this generation. The low line birds fed diet A showed several positive correlations between corticosterone and innate parameters, such as NAb and complement. Because these correlations were moderate, it may suggest that the low line birds of the second generation showed partial neuroendocrine regulation of these parameters due to diet A. Furthermore, all diet A-fed lines showed an either positive (control and to a greater extent low line birds) or negative (high line hens) correlation between corticosterone and the LPS NAb. These results may suggest that the diet A-fed second generation is under more neuroendocrine regulation than the diet B-fed birds and that LPS in diet A may be the most likely candidate to have caused these effects.

In conclusion, our data show that immunomodulation by diet is mostly found in birds with high HPA axis reactivity (in this study, the low line birds), both in a first generation as well as the second subsequent generation, albeit to a lesser extent. Furthermore, diet and a change of diet can induce a corticosterone response in the same genetic line of birds. Birds with low HPA axis reactivity and high Ab production (the high line birds) showed diet effects in production parameters, and to a lesser extent in Ab production. We conclude that animal models with differences in stress reactivity are suitable models for the investigation of individual differences in immunomodulation by diet, but this topic requires further examination.

Acknowledgements

This study was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality (LNV), the Rabobank and Triodos Bank, the Netherlands.

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CHAPTER

VI

Chicken lines divergently selected for Antibody response to SRBC show line-specific differences in sensitivity to immunomodulation by diet, part II: cellular parameters

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Submitted

Abstract

Two diets differing slightly in protein content were found to modulate levels of specific and natural humoral immunity in different manners over two generations of hens genetically selected for a high or low Ab response. Previously, these birds were shown to have differential HPA axis responsiveness. Here we report the effects of these two diets on cellular immune parameters such as monocyte reactivity measured by NO production, *in vitro* proliferation of whole blood leucocytes and PBMC's with the T- and B-cell mitogens ConA and LPS, respectively. In both generations a change of diet enhanced monocyte reactivity in all birds with the High line birds being the most sensitive. *In vitro* whole blood assays showed the most pronounced diet effects on T-cell reactivity. The Low line birds of the first generation showed the greatest effect, but in the second generation all lines were affected by both of the diets. In the PBMC's significant effects were found in the control values, with the effects differing in each generation. These results together with the results found in the whole blood cultures, suggest dietary effects on the intrinsic reactivity of peripheral lymphocytes as well as *in vivo* effects on these cells, the first with rapidly measurable effects and the last activity reflected in our later measurements. The results suggested that a feed change with only minor nutritional differences may induce immunomodulatory effects, and each diet has an unique effect on cellular parameters of innate and adaptive immunity. In the first generation, birds with high HPA axis reactivity showed the greatest sensitivity to nutritional immune modulation. In the subsequent second generation all birds show modulation of the cellular compartment of the immune system, albeit to a different degree. The consequences of these findings are discussed.

Introduction

Dietary deficiencies and supplementation affect the immune system and production parameters of poultry and other food animals (Cook, 1991; Wintergerst et al., 2007; Li et al., 2007) in a positive or negative fashion. More recently, understanding of nutritional effects evolved from macro-nutrients to micro-nutrients and trace elements and other biofunctional components in food. For instance, probiotics are examples of biofunctional components in diet that were claimed to show beneficial effects on the functioning of the immune system (Scharek et al, 2005; Schley and Field, 2002). Apart from energy and building stones, nutrition may be the source of immunogens to which the immune system must become tolerant. Nutrition may provide factors, that either themselves might modulate immune maturation and responses such as PUFA (Stulnig, 2003), or in addition provide ingredients that influence the intestinal microbiota, which in turn may affect antigen exposure, immune maturation and immune responses (Hrncir et al, 2008). It is likely that through these mechanisms nutrition early in life might affect immune competence later in life, the ability to mount an appropriate immune response upon infection, the ability to develop a tolerogenic response to 'self' and to benign environmental antigens, and the development of immunologic disorders (reviewed by Noverr and Huffnagle, 2005).

Albers et al (2005) proposed immune parameters for nutritional intervention studies for humans in three categories (from high suitability to low suitability) that may be useful in detecting immune modulatory effects by nutrition. Vaccine specific antibodies were classified as highly suitable, and *in vitro* lymphocyte proliferation was considered of medium suitability. Previously we showed that using the same feed production method but with raw ingredients from different sources, differences in humoral parameters of the immune system could be detected over two generations of layers (Adriaansen-Tennekes et al, 2009b). Thus, diets with similar caloric values, and similar ingredients, but differing slightly in content of macronutrients and origin of raw materials induced differences in natural and specific antibody responses to model antigens and vaccines in groups of layers fed the different diets. The effects were modest and appeared only in one or two humoral parameters. Furthermore, individual differences in sensitivity to such dietary differences were found in both generations and in the first generation related to HPA axis reactivity.

Here we investigated the effects of measured minor differences between these two diets, on the other major part of the immune system, i.e. cellular parameters of innate and adaptive immunity in two subsequent generations of layer hens. As the *in vitro* lymphocyte stimulation test (LST) was found to be

effective in measuring immunomodulation by diet (Albers et al, 2005), we determined both in vitro lymphocyte proliferation in whole blood as well as isolated peripheral blood mononuclear cells (PBMC's) to be able to differentiate between in situ modulation of lymphocytes as reflected by PBMC's as well as determine the systemic effects as reflected by the whole blood LST's. As monocytes induce subsequent immune modulation, monocyte reactivity was determined by NO production. Birds used in this experiment originated from ISA-Warren brown medium heavy layers that had been divergently selected in the past for high (H), or low (L) antibody responses to sheep red blood cells (SRBC) at 5 days after immunization with SRBC at 5 weeks of age, next to a random bred control (C) line. These birds are also known to differ in the cellular compartments of the immune system as was reviewed previously (Adriaansen-Tennekes et al, 2009b), as well as in HPA axis reactivity (Adriaansen-Tennekes et al, 2009a). Here we report not only minor differences in mineral and trace element contents, but also a change of diet (component(s)) sensitizes the cellular compartment of the immune system.

Materials and Methods

Birds

Two generations of ISA Brown (Warren) medium heavy layer hens from three lines were used. The selection lines were selected for 25 generations prior to this experiment for either high or low primary antibody responses at 5-days after intramuscular immunization (without the use of adjuvant) with 2% packed SRBC at the age of 35-days. The randomly bred third line is the control line, originating from the same parental stock. The experimental first generation consisted of 72 hens evenly divided over the three selection lines, but not immunized with SRBC. In the second offspring generation, 150 chicks, 50 of each line were used, again, not immunized with SRBC.

All experiments were approved by the Animal Welfare committee of Wageningen University.

Experimental Design

The first generation of birds was kept according to routine procedures for layer hens in individual battery cages. The second generation hens were kept at 6 (2 birds of each line per cage) birds per enriched cage (2.28 m^2) in a floor housing system. For both generations the light regimen was 14 h light (0500 to 1900), and temperatures were between 16 and 21°C. At hatch all chicks of the first generation were vaccinated against Marek's disease (Poulvac, Fort Dodge Animal Health, Vaals, The Netherlands), and infectious bronchitis (IB)(MA 5, Nobilis-Intervet, Boxmeer, The Netherlands), IB at d 71 (primer, Poulvac) and d 126 (H52, Nobilis); infectious bursal disease (IBD, Gumboro, D78, Nobilis) at d 22; infectious laryngotracheitis (ILT, Nobilis) at d 86; and New Castle Disease (NCD, clone 30, Nobilis) at d 10, 42, 51, and 114 of age. The second generation birds received vaccinations against Marek's disease and IB at hatch, IBD at d 21 and NCD at d 10, 28 and 40 of age.

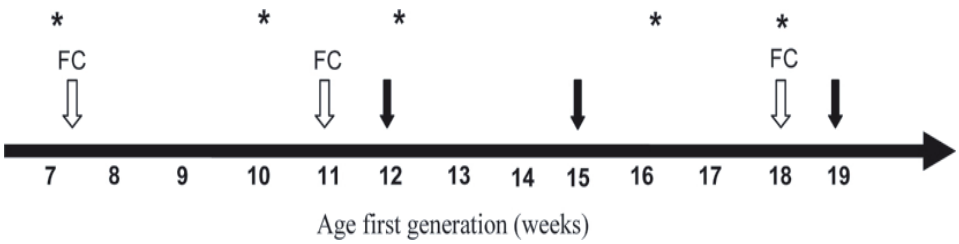

Figure 1. Timeline for the experimental design of the blood sampling for the first generation of hens. At 33 wk of age, the first generation was sampled as well; this is not depicted in the figure. Solid arrows represent blood sampling times, and open arrows represent a feed change (FC). An asterisk (*) represents regular vaccinations.

The first generation was sampled by wing venipuncture according to the schedule as depicted in Figure 1. The second generation was sampled once at eight weeks of age, one week after the change from starter to grower feed, also by wing venipuncture. Blood was collected in a syringe and thereafter divided over a serum tube and heparinized tube. After centrifugation serum and plasma were collected and stored at -20º C until further analysis.

Experimental Feed

All animals received diets that were appropriate for their age, being: starter (from hatch to 6 weeks of age), grower (7-17 weeks) and layer diet (from 18 weeks on). For each appropriate age, two identical experimental diets were made only differing in the cultivation method of the raw ingredients (wheat, barley, triticale, peas, maize and soy). Each raw ingredient, e.g. wheat A, was used for all the produced experimental diets designated diet A. Macronutrient contents of the two experimental diets (A and B) is shown in Table 1. The main difference between diets A and B was protein content (B>A). The first generation of animals received commercial diets from hatch, a conventional diet from 7.5 weeks of age (specially made as the experimental diets were of a different structure (less coarse) than the commercial diet) and the experimental diets from the age of 11 weeks. The second generation received the experimental diets from hatch to the end of the observation period.

Table 1. Macronutrient content in the experimental feeds[1]

	First Generation				Second Generation			
	Grower		Layer		Starter		Grower	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Energy KJ/kg	14480	14610	14487	14302	14729	14882	15231	15102
Ash content g/kg	51	48	113	114	64	51	55	54
Raw fibre g/kg	43	42	36	40	36	34	39	39
Tot. carbohydrates g/kg	546	539	555	538	624	620	585	574
Crude fat g/kg	61	59	69	64	42	42	62	53
Protein g/kg	173	192	147	164	151	164	176	199
Moisture g/kg	126	120	116	120	119	123	122	120
Chloride g/kg	1.7	2.0	1.3	1.4	2.3	2.1	1.9	2.3

[1]Starter feed was given 0 to 6 wk of age, grower feed from 7 to 17 wk of age, and layer from 18 wk of age on. For all of the A diets, the same batches of raw ingredients were used, as was done for all the B diets.

Reagents

Escherichia coli derived lipopolysaccharide (LPS), *Staphylococcus aureus* derived lipoteichoic acid (LTA), Griess reagents' and Concanavalin A (Con A) were from Sigma Chemical Co, St. Louis, MO.

Lymphocyte stimulation test (whole blood)

The lymphocyte stimulation test (LST) was used to determine specific cellular reactivity. Briefly, heparinized blood was diluted 1:30 in RPMI culture medium and 100 µl was added per well of a 96-well flat bottom culture plate. Triplicate cultures were incubated with either 100 µl Con A (for T-cell stimulation, end concentration 20µg/ml), LPS (for B-cell stimulation, end concentration 10µg/ml) or RPMI culture medium (control). All plates were placed for 48 hours in a humidified incubator at 41º C with 5% CO_2. Then 0.4 µCi ^{3}[H]-thymidine was added per well and incubated overnight. Plates were stored at -20º C before harvesting, to prevent clogging of the harvesting apparatus due to the erythrocytes. After thawing of the plates they were harvested onto fiberglass filters, and filters were then counted by liquid scintillation spectroscopy.

Lymphocyte stimulation test (isolated PBMC's)

Triplicate cultures with 10^6 cells per well isolated by Histopaque 1.077 density gradient in 100 µl per well to a 96-well flat bottom culture plate were incubated with either 100 µl Con A (for T-cell stimulation, end concentration 10 µg/ml), LPS (for B-cell stimulation, end concentration 5 µg/ml) or RPMI culture medium (control) for 48 hours in a humidified incubator at 41º C with 5% CO_2. Then 0.4 µCi ^{3}[H]-thymidine was added per well and incubated overnight. Plates were harvested onto fiberglass filters and filters were then counted by liquid scintillation spectroscopy.

Monocyte reactivity expressed as nitrite production

Peripheral white blood leucocytes were isolated from heparinized blood using a Histopaque 1.077 density gradient. Cells were washed two times in RPMI 1640 and then diluted to a $1*10^7$ cell per ml suspension. Triplicate cultures with 10^6 cells per well were incubated in flat bottom 96-wells plates for 72 hours at 41º C with 5% CO_2 with or without (= control) LPS in 200 µl culture medium (end concentration LPS 10µg/ml). After incubation, 50 µl culture medium was extracted from the plates and mixed for 10 minutes at room temperature with 50 µl Griess reagents' in a 96-well flat bottom plate. Extinctions were measured at 540 nm (Multiscan, Labsystems, Helsinki, Finland). Monocyte reactivity was calculated using nitrite calibration and expressed as µM NO production, with a

detection limit of 0.01 μM.

Statistical analysis

The differences in all parameters were analyzed by a three-way ANOVA for the effect of feed, line, time and their interactions using the repeated measurement procedure (with a bird nested within diet and line option), and a two-way ANOVA for the effect of line, feed and their interactions per sample moment. The mean differences due to diet and line were tested with Bonferroni's. Differences between the selection lines were determined by multiple comparison of the means. All data are presented as mean ± SEM. All analyses were according to SAS procedures (SAS, 2003).

Results

First Generation

Lymphocyte stimulation test (whole blood cultures).

The medium control values of all samples (i.e. non-stimulated cells) from the first generation of hens were between 260 – 600 cpm (Table 2). Only after eight weeks consumption of the two experimental diets (wk 19) a numerical difference was seen in the control values, all birds fed diet B, irrespective of line, showed higher counts than the birds fed diet A (p=0.0658, Table 2). Within the diet B fed birds, the selection lines showed significant contrasts at week 15, in which the Control line birds showed the highest control values followed by the Low and High line birds respectively.

Table 2. Average proliferation by lymphocytes from whole blood cultures from High (H), Control (C) and Low (L) line hens fed one of the two experimental diets. All values are actual counts per minute of whole blood cultures with RPMI medium (controls).

		First generation				Second generation
Line	Diet	RM[1]	Wk12	Wk15	Wk19	Wk8
Controls						
H	A	404	551	397	265	1054
C		396	460	425	302	1253
L		398	530	392	272	1171
H	B	422	604	350[b]	312	1177
C		471	559	500[a]	355	1228
L		385	486	373[b]	294	1063
SEM		40.0	99.3	45.7	26.5	119
Main effects[2]						
D		NS	NS	NS	0.0658 B>A	NS
L		NS	NS	NS	NS	NS
DxL		NS	NS	NS	NS	NS
T		<0.0001				
DxT		NS				
LxT		NS				
DxLxT		NS				

[*] Within diet contrast between lines with no common superscript differ significantly: a,b< 0.05. Data are group mean: first generation n=11-13 animals per line/feed; second generation n=21-26 per line/feed.

[1] Least squares means ± SEM of the complete observation period after analysis by a repeated measurement procedure after the change to the experimental diets.

[2] Main effects: D = Diet; L = line; DxL = diet by line interaction; T = time; LxT = line bye time interaction; DxT = diet by time interaction, DxLxT = diet by line by time interaction. NS = no significance, otherwise p value is noted.

Proliferation to LPS, a B-cell mitogen, showed large variance in the first generation of hens with no detectable diet, line or interaction effects (Table 3). Only the Low line birds showed a difference eight weeks after the start of the experimental diets (wk 19), wherein the birds fed diet A showed a significantly

higher cpm than their counterparts fed diet B (p=0.0309). Within the diet A fed birds a contrast between the selection lines was found both four as well as eight weeks after the switch to the experimental feeds.

Table 3. Average proliferation by lymphocytes from whole blood cultures from High (H), Control (C) and Low (L) line hens fed one of the two experimental diets. All values are actual counts per minute of whole blood cultures with LPS.

		First generation					Second generation
Line	Diet	RM[1]	RM SI[1]	Wk12	Wk15	Wk19	Wk8
LPS							
H	A	2056	4.01	4999	767[b]	404[b]	1864[b,y]
C		1744	3.71	3649	1209[b]	374[b]	2902[a,y]
L		2598	5.46	4360	2281[a]	1155[a,y]	1997[a,b,y]
H	B	2397	4.88	4866	1833	492	2801[b,x]
C		2375	4.37	4218	2577	332	4356[a,x]
L		1802	4.13	2755	2391	352[x]	3124[b,x]
SEM		536	0.90	1283	532	254	387
Main effects[2]							
D		NS	NS	NS	NS	NS	0.0003 B>A
L		NS	NS	NS	NS	NS	0.0027 C>L>H
DxL		NS	NS	NS	NS	NS	NS
T		<0.0001	<0.0001				
DxT		NS	NS				
LxT		NS	NS				
DxLxT		NS	NS				

[a] Within diet contrast between lines with no common superscript differ significantly: a,b< 0.05. Statistical difference within line between diets: x,y< 0.05. Data are group mean: first generation n=11-13 animals per line/feed; second generation n=21-26 per line/feed.

[1] Least squares means of the actual counts per minute as well as of the Stimulation Indexes (SI) of the complete observation period after analysis by a repeated measurement procedure after the change to the experimental diets.

[2] Main effects: D = Diet; L = line; DxL = diet by line interaction; T = time; LxT = line bye time interaction; DxT = diet by time interaction, DxLxT = diet by line by time interaction. NS = no significance, otherwise p value is noted.

The *in vitro* T-cell stimulation in the first generation, as represented by the Con A data, showed a three-way interaction between diet, line and time wherein the birds fed diet A, showed numerically higher stimulation to Con A over time when compared to the birds fed diet B (p=0.0637, Table 4). This effect was greatest in the Control and Low line birds, with the last showing significantly higher cpm or stimulation indexes (SI) due to diet A, when compared to their counterparts fed diet B. Within the diet B fed birds a contrast between the selection lines was found one week after the start of the experimental feeds. At wk 15 and wk 19 the contrast between lines was no longer in the diet B fed birds, but now in the diet A fed birds.

Table 4. Average proliferation by lymphocytes from whole blood cultures from High (H), Control (C) and Low (L) line hens fed one of the two experimental diets. All values are actual counts per minute of whole blood cultures with Con A.

		First generation					Second generation
Line	Diet	RM[1]	RM SI[1]	Wk12	Wk15	Wk19	Wk8
Con A							
H	A	30860	93.9	24544	37069^{b}	30970^{a,b}	26165^{y}
C		33131	104.2	32908	43917^{a,b}	22570^{b}	29424^{y}
L		40292^{y}	119.6^{y}	30321^{y}	52100^{a,y}	38458^{a}	28854^{y}
H	B	32416	89.1	31804^{a}	40248	25199	13215^{x}
C		39901	76.1	28276^{a}	42148	25547	13785^{x}
L		28048^{x}	82.4^{x}	17537^{b,x}	35360^{x}	29427	14090^{x}
SEM		4004	12.7	4286	5527	3863	2094
Main effects[2]							
D		NS	0.0282 A>B	NS	NS	NS	<0.0001 A>B
L		NS	NS	NS	NS	0.0439 L>H>C	NS
DxL		NS	NS	0.0727	NS	NS	NS
T		<0.0001	<0.0001				
DxT		NS	NS				
LxT		0.0022	0.0006				
DxLxT		0.0637	0.0996				

* Within diet contrast between lines with no common superscript differ significantly: a,b< 0.05. Statistical difference within line between diets: x,y< 0.05. Data are group mean: first generation n=11-13 animals per line/feed; second generation n=21-26 per line/feed.

[1]Least squares means of the actual counts per minute as well as of the Stimulation Indexes (SI) of the complete observation period after analysis by a repeated measurement procedure after the change to the experimental diets.

[2]Main effects: D = Diet; L = line; DxL = diet by line interaction; T = time; LxT = line bye time interaction; DxT = diet by time interaction, DxLxT = diet by line by time interaction. NS = no significance, otherwise p value is noted.

Lymphocyte stimulation test (isolated PBMC).

Over time a consistent diet effect was seen wherein the birds fed diet B had higher control values when compared with the birds fed diet A (RM, p<0.0001, Table 5). This effect was greatest respectively in the Low, Control and then High line birds and declined over time.

The cpm's of the cultures with LPS were low and comparable to the medium control values (data not shown). No discernable differences were measured.

Overall the counts per minute were low and close to control values when the PBMC's were cultured with Con A (data not shown). Only the High line birds showed a difference due to diet, the birds fed diet B showed significantly higher cpm's compared to the birds fed diet A.

Monocyte reactivity.

Monocytes were cultured with LPS to analyze the LPS induced NO production capacity *ex vivo*. The medium controls showed a significant diet by

Table 5. Average a-specific proliferation of lymphocytes from isolated PBMC's in High (H), Control (C) and Low (L) line hens on the two experimental feeds. All values are actual counts per minute of medium control cultures.

		First generation				Second generation
Line	Diet	RM[1]	Wk12	Wk15	Wk19	Wk8
H	A	872^{y}	545^{y}	730^{a}	1327^{y}	$2392^{a,y}$
C		771^{y}	545^{y}	$439^{b,y}$	1327	1137^{b}
L		811^{y}	654^{y}	$521^{a,b}$	1275	1672^{b}
H	B	1046^{x}	789^{x}	709	1626^{x}	1503^{x}
C		976^{x}	760^{x}	722^{x}	1444	919
L		1003^{x}	840^{x}	698	1458	1238
SEM		53.0	74.3	85.2	123	288
Main effects[2]						
D		<0.0001 B>A	0.0008 B>A	0.0398 B>A	0.0506 B>A	0.0315 A>B
L		NS	NS	NS	NS	0.0104 H>L>C
DxL		NS	NS	NS	NS	NS
T		<0.0001				
DxT		NS				
LxT		NS				
DxLxT		NS				

* Within diet contrast between lines with no common superscript differ significantly: a,b< 0.05. Statistical difference within line between diets: x,y< 0.05. Data are group mean: first generation n=11-13 animals per line/feed; second generation n=21-26 per line/feed.

[1] Least squares means ± SEM of the complete observation period after analysis by a repeated measurement procedure after the change to the experimental diets.

[2] Main effects: D = Diet; L = line; DxL = diet by line interaction; T = time; LxT = line bye time interaction; DxT = diet by time interaction, DxLxT = diet by line by time interaction. NS = no significance, otherwise p value is noted.

time interaction (p=0.0003, Table 6). A diet effect was found at wk 12, in which all birds fed diet A showed higher NO production than the diet B fed birds. After each feed change (wk12 and wk19), all medium controls, irrespective of line or diet, showed enhanced NO production (Figure 2). At week 15, four weeks after the switch to the experimental diets, all control values were below the detection limit. The High and Control line birds fed diet A showed significantly higher NO production than the hens fed diet B after the first diet change, which was the switch to the experimental diets.

The LPS induced NO production by monocytes from the tested birds showed a significant interaction between diet, time and line in the first generation (p=0.0471, Table 6) with a consistent difference in reactivity of the selection lines, the Control line birds showed the highest reactivity, the Low line the least with the High line in between (Figure 2).

Table 6. Nitric oxide (μM) production during or after 8 weeks (respectively first and second generation) of feeding with the experimental diets from High (H), Control (C) and Low (L) line hens on the two experimental feeds. Control values are from unstimulated PBMC cultures.

		First generation[1]		Second generation[2]	
Line	Diet	Controls	LPS	Controls	LPS
H	A	1.57^{a,y}	12.09	0.19	15.99^{a,y}
C		1.55^{a}	12.79	0.03	8.96^{b,y}
L		1.20^{b}	8.67	0.09	6.60^{b}
H	B	1.06^{x}	12.58^{a}	0.10	2.62^{x}
C		1.25	12.11^{a,b}	0.03	2.30^{x}
L		1.16	6.99^{b}	0.37	3.03
SEM		0.15	2.22	0.18	2.38
Main effects[3]					
D		0.0206 A>B	NS	NS	0.0001 A>B
L		NS	0.0683 C>H>L	NS	NS
DxL		NS	NS	NS	NS
T		<0.0001	0.0005		
DxT		0.0003	NS		
LxT		NS	NS		
DxLxT		NS	0.0471		

* Within diet contrast between lines with no common superscript differ significantly: a,b< 0.05. Statistical difference within line between diets: x,y< 0.05. Data are group mean: first generation n=11-13 animals per line/feed; second generation n=21-26 per line/feed.

[1] Least squares means of the complete observation period after analysis by a repeated measurement procedure. Data are group mean: first generation n=11-13 animals per line/feed.

[2] Average NO production after eight weeks of feeding and one week after the change from starter to grower feeds. Data are group mean: second generation n=21-26 per line/feed.

[3] Main effects: D = Diet; L = line; DxL = diet by line interaction; T = time; LxT = line bye time interaction; DxT = diet by time interaction, DxLxT = diet by line by time interaction. NS = no significance, otherwise p value is noted.

Correlations between corticosterone levels and immune parameters.

Previously we have presented the different corticosterone levels during this experiment (Adriaansen-Tennekes et al, 2009b). In summary: a diet effect was found after the change to the experimental diets wherein the diet A fed birds showed higher corticosterone levels than the diet B fed birds. The Low line hens fed diet A showed significantly higher corticosterone levels one week after the change to the experimental diets, when compared with the Low line birds fed diet B. Thereafter the corticosterone levels of the Low line birds fed diet A were consistently higher when compared to the Low line birds fed diet B. No differences were measured in the other two selection lines.

One week after the change to the experimental diets, some correlations with the corticosterone levels were found in the Low line birds. There was a strong positive correlation between corticosterone and PBMC LST with ConA ($r = 0.75362$, $p = 0.0118$). In contrast the Low line birds fed diet B showed a negative

correlation with the PBMC LST with ConA (r = -0.62126, p = 0.0552). The Control line hens fed diet B showed a moderate correlation with the PBMC LST with LPS (r = 0.62527, p = 0.0297), and the Control line hens fed diet B showed a positive correlation with the monocyte controls (r = 0.58122, p = 0.0475). The High line birds fed diet A showed a strong positive correlation between the monocytes stimulated with LPS and corticosterone (r = 0.85104, p = 0.0018).

After four weeks of feeding the experimental diets, several consistent correlations were found differing between the lines. The High line birds fed diet A showed significant correlations with all whole blood (WB) parameters (respectively WB control, WB LPS and WB Con A: r = 0.57966, - 0.53284 and – 0.61190). The Control line birds fed diet B showed significant negative correlations with the measured corticosterone levels with the PBMC controls (- 0.57373) and ConA stimulated (- 0.52654) lymphocytes. The Low line hens fed diet B showed negative correlations with corticosterone and all PBMC proliferations, independent of the used mitogen (respectively control, LPS and Con A: r = - 0.72771, - 0.69894 and – 0.53640).

At week 19, eight weeks after starting the experimental diets, again the Low line birds fed diet B showed the most correlations with corticosterone, albeit with different parameters. Now the positive correlations were with the mitogen stimulated whole blood (WB) proliferations (respectively LPS and ConA: 0.83837, 0.54338) and the PBMC proliferation with LPS (0.59910). The Control line hens fed diet B showed strong positive correlations between corticosterone and the monocyte parameters (respectively controls and LPS: r = 0.70473 and 0.63001).

Second Generation

Lymphocyte stimulation test (whole blood cultures).

The medium control values of the second generation samples were all approximately 1000 cpm, with no differences due to line or diet. These relatively high control values (Table 2) could be explained by the floor housing of this generation (relatively 'dirty' housing conditions).

The week after the change from starter to grower feeds (wk 8), a significant diet effect was seen wherein all the hens on diet B showed a higher proliferative response to LPS (p=0.0003, Table 3). This difference was significant for the Control and Low line birds. Furthermore, a significant line effect was seen (p=0.0027) wherein the Control line showed the highest cpm followed respectively by the Low and High line birds.

The T-cell response of the second generation of birds showed significantly

higher proliferation in the hens fed diet A ($p<0.0001$, Table 4), being the week after the feed change.

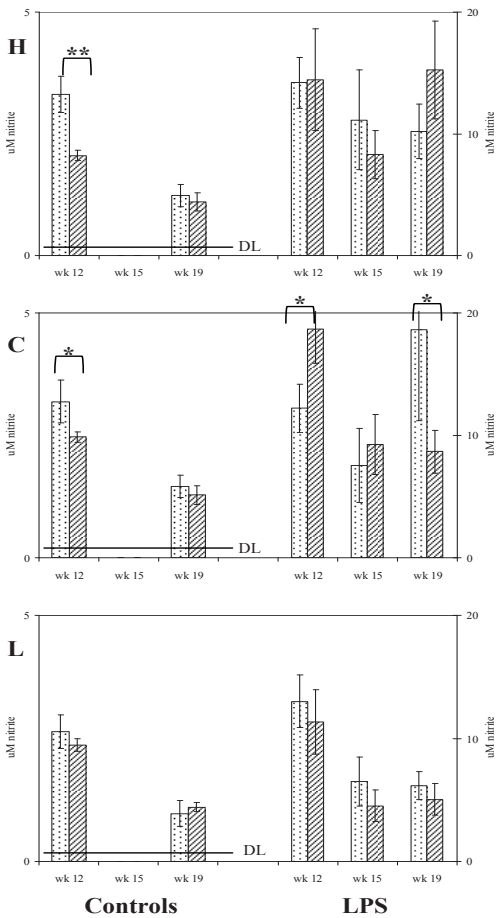

Figure 2. NO production of monocytes in isolated PBMC cultured in RPMI or with LPS from High (H), Control (C) and Low (L) line hens. The dotted bars represent the diet A fed animals, the lined bars represent the diet B fed animals. Data are mean ± SEM. In the first generation n=11-13 animals per line/feed. Statistical difference within line between diets: * $p< 0.05$; ** $p< 0.01$. DL represents detection limit of the assay.

Lymphocyte stimulation test (isolated PBMC).

The second generation of birds had higher control values when compared with the first generation, again this may be the result of relatively dirty housing

conditions (Table 5). All birds fed diet A consistently showed significantly higher cpm when compared with their counterparts fed diet B (p=0.0315). Next to the diet effect a significant line effect was found as well (p=0.0104), with the High line birds showing the greatest cpm, followed by the Low and Control line birds respectively.

The counts of the LPS cultures were low and comparable to the medium control values. Diet A showed significantly higher counts in all lines when compared with the counterparts on diet B (p=0.0055, data not shown).

The T-cell proliferation showed greater proliferation due to diet A (p=0.0271, data not shown) and a significant line difference (H>C>L, p<0.0001).

Monocyte reactivity.

When regarding the medium control values in the second generation (Table 6), regardless of the chicken line and time point, all values were low and close to zero.

In the second generation the LPS stimulated NO production the week after the birds switched from the first experimental diet ("Starter") to the second diet ("Grower") was significantly higher in the diet A fed birds. All hens, regardless of line, fed diet A showed greater LPS stimulated NO production values (p=0.0001, Table 6). The highest values are seen in all the diet A fed birds wherein the H-line birds showed the highest values, the lowest values were seen in the L-line birds fed diet A, and the C-line hens fed diet A in between. The birds fed diet B showed no differences due to the selection lines, all hens showed similar NO production.

Correlations between corticosterone levels and immune parameters.

No diet effects in corticosterone levels were found in the second generation. The greater HPA axis reactivity of the Low line animals was reproduced.

There were few positive correlations between the measured immune parameters and the corticosterone levels. The Control line birds fed diet A showed correlations with respectively WB controls and PBMC with Con A (0.58595 and 0.44292). The Low line hens fed diet B showed a strong positive correlation with the monocyte controls (r = 0.71213, p = 0.0001).

Discussion

In the present study two nutritionally virtually identical diets were shown to display differential effects on cellular immune parameters representing the innate and adaptive immune system in two consecutive generations of layer hens divergently selected for their antibody response against SRBC. The effects of the two different diets were measured after each feed change in both generations. Previously we showed that small differences in the two diets had significant modulatory effects on innate (NAb) and specific (vaccine Ab) humoral immune parameters in these two generations of layers (Adriaansen-Tennekes et al, 2009b).

When the effects on both generations are taken together, each feed change induced a profound change in immune responsiveness as was reflected in the altered monocyte reactivity and the whole blood lymphocyte stimulation *ex vivo*. In the first generation, birds fed diet A, showed higher monocyte reactivity and T-cell responsiveness ex vivo. The second generation showed these same effects for the diet A-fed birds, as well as an increased B-cell responsiveness in the diet B-fed birds after the feed change. The reactivity of the isolated PMBC's to T- and B-cell mitogens was not affected by the feed changes, suggesting no *in situ* change in the capacity of the lymphocytes to respond to antigenic challenges. Interestingly, the culture medium controls of the PBMC's LST did differ significantly and were reversed due to the two diets over the two generations, suggesting an induced differential responsive state of the lymphocytes *in situ*. Possibly, a part of the nutritional effects rest on components present in plasma. This was also suggested by Finamore et al (2004), where isolated rat lymphocytes cultured with rat serum did show differences, but when cultured with fetal calf serum no differences were found. The present results with two comparable layer feeds on cellular immunity are in line with our previously reported effects found with humoral parameters, i.e. levels of natural antibodies and vaccine specific antibodies were affected by diet.

The Low line hens fed diet A, but not the other lines fed diet A or diet B, showed a marked corticosterone response to the change to the experimental diet at the age of 12 weeks. After the change to the layer diet eight weeks later, again only the Low line birds showed an increase in corticosterone levels, albeit to a lesser extent. This lower corticosterone response to the second feed change in the low line birds is comparable to a second exposure to the same stressor (Edgar et al, 2003; Richards et al, 2006).

After each feed change, all *in situ* monocyte reactivity as reflected by the culture medium control NO production, was enhanced by both diets, albeit in

different magnitudes in the selection lines. The control measurements between feed changes were all below detection limit (15 weeks of age), indicating that the *in situ* response state of the monocytes was rapidly normalized. Lim et al (2007) reported *in situ* activation of peritoneal macrophages due to elevated corticosterone *in vivo*, with stimulating as well as inhibiting effects depending on the concentration of corticosterone used *in vitro*. In an experiment by Smyth et al (2004) pretreatment of macrophages with corticosterone elevated subsequent NO production induced by LPS. We found correlations between the monocyte parameters and the corticosterone levels in the High and Control line hens only after feed changes. Dietary factors such as PUFA, β-glucans and LPS have also been shown to enhance LPS induced NO production by macrophages (Stulnig, 2003; Akramienė et al, 2007). Whether our results are the effect of corticosterone and/or dietary factors remains to be established.

Our PBMC cultures showed no differences in stimulation by ConA or LPS, reflecting no change in the *in situ* immune responsiveness capacity of the T- and B-lymphocytes to respond. On the other hand a significant difference between control values was seen due to the diets. The diet A fed birds showed lower control values when compared to the diet B fed hens, and this difference decreased over time. A similar effect was seen by Edgar et al (2003) after chronic mild stress, wherein the stressed animals showed lower control values as well. Finamore et al (2004) showed the same difference in control values as found in our experiment in rats fed two nutritionally complete but different diets. Especially the current Control and Low line birds showed several correlations between the corticosterone levels and the PBMC parameters, suggesting that corticosterone played a role in the dietary effects on these parameters. Furthermore, these results suggest that the PBMC control values may reflect environmental conditions, next to the well known daily hormonal influences.

Whole blood cultures were expected to reflect systemic effects due to the diets. The present results showed enhanced proliferation to Con A in the diet A fed birds when compared to the diet B fed birds with the greatest differences between the diets found in the Low line birds. Enhancement of the Con A induced proliferative response has been reported in relation to stress by several groups (Leandro et al, 2006; Levay et al, 2006; Satoh et al, 2006). However, Satoh et al (2006) found no effects due to stress in the LPS induced proliferative response, which was also found here. Dietary components, such as glucans, fish oil and others have been found to enhance lymphocyte proliferation when supplemented to the diets (Mao et al, 2005; Wang et al, 2008; Stulnig, 2003; Shaikh and Edidin, 2006; Volman et al, 2008). As was true for the isolated cells, here too several correlations were found between the corticosterone levels and the whole blood

parameters. These correlations were in the High and Low line birds, suggesting systemic effects of the diets based on corticosterone present in plasma. Again, the exact role of corticosterone and the dietary components remains to be established.

Although the diets used in this experiment showed minor differences in for instance levels of protein, it is unlikely that the observed effects were the result of such minor differences. Generally, effects of dietary components on the immune system are usually determined by excessive supplementation of the component to the diet and/or by artificially exaggerated differences between diets. It is conceivable that effects on immune responses may rest on synergistic (or cancelling) effects by minor differences in dietary components as reported previously by Cheng et al (2006). As there were small, but significant differences in corticosterone levels between the Low line birds fed the two diets and several correlations between the corticosterone levels and the immune parameters were found, this seems the most likely mechanism (partially) causing the found effects, although synergistic/cancelling effects with dietary components can not be ruled out.

It is possible that the corticosterone levels were elevated due to both diets as the measured levels in our experiments were higher than previously reported levels due to immunization and cold stress in these selection lines (Siegel et al, 1992; Hangalapura et al, 2004). This may explain the elevation of *in situ* monocyte NO production in all birds of the first generation after each feed change in both diets. Furthermore, the differences in HPA axis reactivity between these selection lines, as well as the observed correlations, also support the strong role of corticosterone in the found effects as the most pronounced effects were found in the Low line hens. Levay et al (2006) showed that rats with high HPA axis reactivity exhibited greater corticosterone responses to stress as well as higher Con A proliferation after stress when compared to rats with low HPA axis reactivity.

In the present paper, the second generation birds showed more divergence in the cellular immune response when compared with the previously presented antibody responses in these birds (Adriaansen-Tennekes et al, 2009b). Neuroendocrine investigation of various selection lines often show no differences under normal, stress free conditions, but differences between the lines occur when the systems are challenged (De Boer et al, 2003). It is tempting to speculate that the enhanced difference in the second generation may also be the result of the feed change prior to the sampling procedure, which may have acted as a trigger for the cellular compartment. Alternatively, maternal nutritional effects on offspring were reported before (Symonds et al, 2007), but mostly in models with

exaggerated differences between the used diets/treatments. Also epigenetic effects of stress in mothers have resulted in altered HPA axis reactivity in the offspring (Clinton et al, 2008). In our model the differences between the diets were small. Most likely a combination of nutritional and maternal factors have caused the found differences in the cellular parameters of the second generation.

Individual differences in sensitivity to feed or food components has been suggested previously (Grimble, 2001; Vicennati et al, 2002; Desiere, 2004). The use of the selection lines here was critically important in examining which individuals may be more sensitive to feed induced immunomodulation. In the first generation, the measured effects were often found only in one or two of the three selection lines, mostly in the birds with high HPA axis reactivity, i.e. the Low line birds. The found effects were probably (at least partially) regulated by corticosterone as indicated by the significant correlations. The second generation, wherein the feed change induced the same modulatory effects in all three of the selection lines, albeit in different magnitudes, suggested an additional immune modulatory effect caused by maternal nutrition possibly through (epi-)genetic factors. The feed changes in our experiment induced triggering of the immune system. We propose that the HPA axis plays an important role in modulation of the immune system by diet, which depends on the genetic background of the bird. Selection lines provide a suitable model to examine the exact nature of individual differences in feed or nutrient sensitivity on immunity as well as HPA axis reactivity in poultry.

Acknowledgements

This study was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality (LNV), the Rabobank and Triodos Bank, the Netherlands.

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CHAPTER

VII

Nutritionally induced early phase and late phase immunomodulation during the immune response to KLH in divergently selected chicken lines

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Concept

Abstract

In neuroendocrine research often differences in neuroendocrine regulation are only found after triggering of the animal, thus found in the reactivity of the response. Antibody (Ab) levels and the antibody response are the downstream result of activation of the immune system. We hypothesized that in birds receiving different diets and showing no measurable differences in humoral immunity may show differences in humoral parameters when immunologically triggered. Using selection lines differing in Ab production, we investigated potential differences in humoral reactivity to the inoculation with KLH. Innate (classical and alternative complement activity, Natural Ab's) humoral parameters, as well as adaptive (vaccine specific Ab's) humoral parameters were determined. Cellular parameters reflecting innate (monocyte reactivity in NO production) and adaptive immunity (proliferation) were also determined. To evaluate a potential role of the neuroendocrine system, corticosterone levels were measured as well. While the primary KLH SpAb responses of the selection lines only differed slightly between the selection line, the three selection lines all showed line-specific corticosterone responses. The greatest effects in corticosterone responses were found in the Low line birds, with high HPA axis reactivity. All humoral parameters, except for APW, showed significant three way interactions between line by diet by time, reflecting altered line-specific differences in the kinetics of the response to the KLH inoculation. Cellular parameters were differentially affected during the different phases of the immune response to KLH. The Low line birds showed numerous correlations between immune parameters and corticosterone levels, suggesting partial regulation of the immune response by corticosterone. In conclusion, to detect potential immunomodulation of humoral parameters by diet, a trigger is necessary, and selection lines differing in Ab production as well as HPA axis reactivity, show line-specific modulation by diet.

Introduction

Nutritional immunomodulation contributes to survival of the offspring in ever changing environmental conditions (Burdge et al, 2007). The risk of disease is often associated with genetic polymorphisms, but the effect is dependent on dietary intake and nutritional status. Individual differences in sensitivity to nutritional (immuno-) modulation have been reviewed previously (Grimble, 2001; Desiere, 2004). Antioxidant status and genetic background of the individual have been reported of importance in the explanation for individual differences of effectiveness of immunonutrition.

Mostly dietary components are investigated by supplementing them to diets in exaggerated amounts. Only few studies are reported wherein two nutritionally complete, but slightly different diets are compared. Millet et al (2005) found no differences due to two comparable but different diets on immunoglobulin levels. Finamore et al (2004) only found differences in proliferative capacity when the animals were compromised by low protein diets. Previously we have reported differences in immunomodulation due to two nutritionally complete but slightly different diets especially on cellular parameters and to a lesser extent also on some humoral parameters of the immune system (Adriaansen-Tennekes et al; 2009a; 2009b *submitted*). Cellular parameters were consistently found to be more reactive (proliferation and NO production) to the diets and diet changes than the humoral parameters (antibodies and complement) were. In a first generation Low line birds, with low antibody production and high HPA axis reactivity (corticosterone production), showed the greatest sensitivity to nutritional immunomodulation. In the subsequent second generation all selection lines showed effects in the cellular parameters, albeit in different magnitudes.

Often in animal models with differential neuroendocrine or behavioral responses no differences are found under stress-free, baseline conditions but these differences do occur when the animals are triggered, for example by a stressor (de Boer et al, 2003; Minozzi et al, 2008). Here we postulate that under normal conditions, nutritionally induced differences on humoral parameters of immunity may be difficult to detect, depending on the immune parameters used, requiring a comprehensive analysis of humoral and cellular compartments of both innate and adaptive immunity. It is in the reactivity to a trigger that potential differences in immune response capacity may be found.

In this study we hypothesized that a trigger was necessary to be able to measure potential differences in the humoral and cellular compartment of the immune system in the second generation of birds fed these diets. As all selection

lines in the second generation showed immunomodulation in cellular parameters, we hypothesized that a second generation would be most likely to show (line-specific) humoral effects due to a trigger. To trigger the immune system, we used a trigger that is known to activate the immune system as well as the neuroendocrine system as our previous work showed partial regulation of the found differences due to differences in HPA axis reactivity (Adriaansen-Tennekes et al, 2009b). The immune response to the neo-antigen KLH was followed measuring humoral parameters, as well as cellular parameters and the stress marker, corticosterone. Sijben et al (2001) showed earlier that the response to an immunization is presented in three stages: 1) early stage with the peak of the Ab response around 5 days after immunization (0 to 7 days after immunization), plateau phase (8 – 14 days after immunization) and late phase (from day 15 onward). As differences in the cellular parameters were found in a second generation, we propose differences in cellular reactivity may be reflected in humoral parameters during the immune response to the neo-antigen KLH.

Materials and Methods

Birds

Hens originating from a second generation of ISA Brown (Warren) medium heavy layer hens fed two diets differing only in origin of the raw ingredients, from three lines were used. The lines were selected for 25 generations prior to this experiment for either high or low primary antibody responses at 5 days after intramuscular immunization (without the use of adjuvant) with SRBC at the age of 35 days. The randomly bred third line is the control line, originating from the same parental stock. The offspring generation, 150 chicks, 50 of each line were used, not immunized with SRBC.

All experiments were approved by the Animal Welfare committee of Wageningen University.

Experimental design

The hens were kept at 6 (2 birds of each line per cage) birds per enriched cage (2.28 m^2) in a floor housing system. The light regimen was 14 h light (0500 to 1900), and temperatures were between 16 and 21°C. At hatch all chicks received vaccinations against Marek's disease and IB, IBD at 3 wks and NCD at d 10, 28 and 40 of age.

When the chicks were 9 weeks of age, all animals received an intramuscular injection in the breast muscle with 1 mg of keyhole limpet hemocyanin (KLH) in 1 ml phosphate buffered saline (PBS, pH 7.2) per bird. The birds were sampled by wing venipuncture at eight weeks of age, and weekly during four weeks after the intramuscular injection with KLH. Blood was collected in a syringe and thereafter divided over a serum tube and heparinized tube. After centrifugation serum and plasma were collected and stored at -20º C until further analysis.

Experimental Feed

All animals received diets that were appropriate for their age, being: starter (from hatch to 6 weeks of age) and grower (7-17 weeks) diet. For each appropriate age, two identical experimental diets were made only differing in the cultivation method of the raw ingredients (wheat, barley, triticale, peas, maize and soy). Each raw ingredient, e.g. wheat A, was used for all the produced experimental diets designated diet A. Diet composition, and macronutrient contents have previously been reported (Adriaansen-Tennekes et al, 2009b). The main difference between diets A and B was protein content (B>A). The birds received the experimental diets from hatch to the end of the observation

period.

Reagents

Escherichia coli derived lipopolysaccharide (LPS) and *Staphylococcus aureus* derived lipoteichoic acid (LTA) were from Sigma Chemical Co, St. Louis, MO. Keyhole limpet hemocyanin (KLH) was purchased from Cal Biochem - Novabiochem Co., La Jolla, CA. Griess reagents' and Concanavalin A (Con A) were from Sigma Chemical Co, St. Louis, MO.

Specific and natural antibodies

Levels of natural and specific antibodies (respectively NAb and SpAb) were determined by indirect two-step enzyme-linked immunosorbent assays (ELISA) (Parmentier et al, 2004). In short, 96-well medium binding plates were coated overnight at 4° C with LPS, LTA, KLH, NCD or IBD. The next day, two-fold diluted plasma was added and incubated for one hour at room temperature. Detection of binding of antibodies to antigen was performed with horse radish peroxidase labeled rabbit anti-chicken IgG_{H+L}conjugate (RACh/IgG_{H+L}/PO, Nordic, Tilburg, The Netherlands) and the substrate tetramethylbenzidine (TMB) and stopped with H_2O_2. Extinction was measured at 450 nm (Multiscan, Labsystems, Helsinki, Finland). The titers were expressed as the 2log values of the highest dilution giving a positive reaction. As small effects are expected due to diet, delta titers were calculated by increase/decrease in titer between first measurement and further measurements to determine immune responsiveness (e.g. titer at wk11 - wk10, titer at wk13 - wk10).

Hemolytic Complement Assay

Serum from all birds was diluted serially in 96-well flat bottom plates and incubated with either sensitized bovine or sheep erythrocytes to measure alternative (APW) or classical complement pathway (CPW), respectively. Every 30 minutes of the 1.5 hours incubation period the plates were shaken. Lysis (the amount of light scattering by erythrocytes) was measured by measuring at the wavelength of 690 nm. The extinctions were transformed by a log-log equation (Von Krogh, 1916), and the hemolytic titer was expressed as the titer with 50% lysis of red bloods cells (CH50 U/ml).

Lymphocyte stimulation test (whole blood)

The lymphocyte stimulation test (LST) was used to determine specific cellular reactivity. Briefly, heparinized blood was diluted 1:30 in RPMI culture medium and 100 µl was added per well of a 96-well flat bottom culture plate.

Triplicates were incubated with either 100 µl Con A (for T-cell stimulation), LPS (for B-cell stimulation) or RPMI culture medium (control) for 48 hours in a humidified incubator at 41º C with 5% CO_2. Then 0.4 µCi ^{3}[H]-thymidine was added per well and incubated overnight. Plates were stored at -20º C before harvesting. After thawing of the plates they were harvested onto fiberglass filters, and filters were then counted by liquid scintillation spectroscopy.

Lymphocyte stimulation test (isolated PBMC's)

Triplicate cultures with 106 isolated cells in 100 µl per well to a 96-well flat bottom culture plate were incubated with either 100 µl Con A (for T-cell stimulation), LPS (for B-cell stimulation) or RPMI culture medium (control) for 48 hours in a humidified incubator at 41º C with 5% CO_2. Then 0.4 µCi ^{3}[H]-thymidine was added per well and incubated overnight. Plates were stored at -20º C before harvesting. After thawing of the plates, they were harvested onto fiberglass filters and filters were then counted by liquid scintillation spectroscopy.

Monocyte reactivity expressed as nitrite production

Peripheral white blood leucocytes were isolated from heparinized blood using a Histopaque 1.077 gradient. Cells were washed two times in RPMI 1640 and then diluted to a $1*10^7$ cell per ml suspension. Triplicate cultures with 10^6 cells per well were incubated in flat bottom 96-wells plates for 72 hours at 41º C with 5% CO_2 with or without (= control) LPS in 200 µl culture medium. After incubation, 50 µl culture medium was extracted from the plates and mixed for 10 minutes at room temperature with 50 µl Griess reagents' in a 96-well flat bottom plate. Extinctions were measured at 540 nm (Multiscan, Labsystems, Helsinki, Finland). Monocyte reactivity was calculated using a nitrite calibration and expressed as µM NO production, with detection limit of 0.01 µM.

Plasma corticosterone

Plasma corticosterone was determined using a radioimmunoassay kit (IDS, Inc., Bolton, UK) with a detection interval of 0 – 2000 ng/ml.

Statistical analysis

The differences in all parameters were analyzed by a three-way ANOVA for the effect of diet, line, time and their interactions using the repeated measurement procedure (with a bird nested within diet and line option). The mean differences due to diet and line were tested with Bonferroni's. Differences between the selection lines were determined by multiple comparison of the

means. Correlations between parameters were calculated using Pearson's correlation coefficients. All data are presented as mean ± SEM. All analyses were according to SAS procedures (SAS, 2003).

Results

Plasma corticosterone.

Corticosterone levels measured throughout the observation period showed a significant interaction between diet, line and time (Table 1). The time-related changes in corticosterone levels after the KLH inoculation are depicted in Figure 1. The High line birds fed the two different diets showed similar corticosterone responses to the KLH inoculation. The Control line hens fed diet B consistently showed slightly higher corticosterone levels throughout the observation period. Only the Low line hens showed significant differences in the corticosterone response to the KLH inoculation, which was first higher in the diet A fed birds and after week 12 was higher in the diet B fed birds. The measurements at week 12 and 13 showed significant diet effects (respectively: $p = 0.0728$, $p = 0.0186$) wherein all birds fed diet B were found to have higher corticosterone levels than the diet A fed hens.

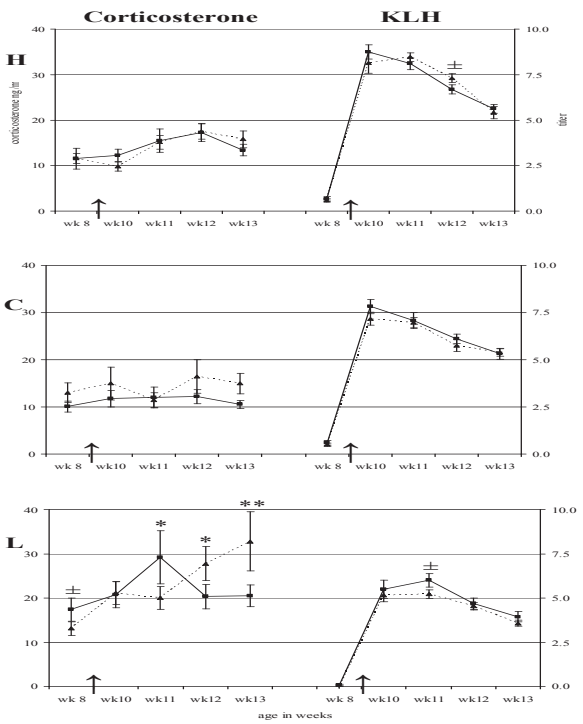

Figure 1. Corticosterone (ng/ml) levels and KLH SpAb in the High (H), Control (C) and Low (L) line animals on respectively diet A (■, solid line) or diet B (▲, hatched line). Data are mean ± SEM. Group size is n=21-26 per line/diet. ↑ arrow indicates KLH inoculation. ± $p<0.1$; * $p<0.05$; ** $p<0.01$.

Table 1. Measured corticosterone levels (ng/ml) and humoral immune parameters[1] measured weekly (4x) in High (H), Control (C), and Low (L) line hens after KLH inoculation.

Line[2]	Diet	B	APW	CPW	NCD	IBD	LTA	LPS	KLH
H	A	13.9^{b}	128.5^{a}	$394.3^{a,x}$	0.69	1.75^{b}	3.41^{a}	$4.09^{a,b,x}$	6.60^{a}
C		11.4^{b}	106.3^{b}	361.4^{a}	0.43	$2.98^{a,x}$	$1.51^{b,x}$	4.45^{a}	5.99^{b}
L		21.6^{a}	92.4^{b}	296.5^{b}	0.37	2.60^{a}	$2.23^{b,x}$	3.51^{b}	4.96^{c}
H	B	14.0^{b}	132.0^{a}	$334.8^{a,y}$	0.73^{a}	1.93^{b}	3.89	$5.46^{a,y}$	6.74^{a}
C		14.4^{b}	117.3^{a}	323.7^{a}	0.31^{b}	$1.86^{b,y}$	3.70^{y}	3.83^{b}	5.74^{b}
L		23.2^{a}	87.0^{b}	269.7^{b}	$0.43^{a,b}$	2.95^{a}	3.44^{y}	3.90^{b}	4.68^{c}
SEM		1.56	9.05	22.8	0.16	0.36	0.38	0.35	0.25
Main Effects[3]									
Diet		NS	NS	0.0286 A>B	NS	NS	<0.0001 B>A	NS	NS
Line		<0.0001 L>H>C	<0.0001 H>C>L	0.0014 H>C>L	0.0520 H>L>C	0.0309 L>C>H	0.0155 H>L>C	0.0092 H>C>L	<0.0001 H>C>L
DxL		NS	NS	NS	NS	0.0924	0.0825	0.0217	NS
Time		0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
DxT		0.0061	NS	<0.0001	0.0013	NS	0.0271	<0.0001	NS
LxT		0.0495	0.0602	<0.0001	0.0545	<0.0001	0.0131	<0.0001	<0.0001
DxLxT		0.0862	NS	NS	<0.0001	0.0118	<0.0001	0.0021	0.0870

*B = corticosterone levels; APW = alternative complement activity; CPW = classical complement activity; NCD = vaccine specific Ab to NCD; IBD = vaccine specific Ab to IBD; LTA = Natural Ab binding to LTA; LPS = Natural Ab binding to LPS; KLH = Specific Ab to KLH.

[1] Data are group (least square) mean of repeated measures, n=21-26 per line/feed. Within diet contrast between lines with no common superscript differ significantly: a,b,c< 0.05. Statistical difference within line between diets: x,y< 0.05. Titers are log2 of the reciprocal of the antibody dilution. The delta titer is increase/decrease in absolute titer between the first measurement and all following measurements (e.g. wk 13 - wk 10).

[2] Line: H = High line; C = Control line, L = Low line.

[3] Main effects based on Main Effect Means: D = Diet; L = line; DxL = diet by line interaction; T = time; LxT = line bye time interaction; DxT = diet by time interaction, DxLxT = diet by line by time interaction. NS = no significance, otherwise p value is noted.

Humoral immune parameters.

The KLH SpAb's reflect the primary immune response to the KLH inoculation in which a three way interaction with diet by line by time was found (Table 1). The High and Low line birds show reversed effects due to the diets: High line birds fed diet B showed higher SpAb's while the Low line birds fed diet

A showed more KLH SpAb's when compared with their respective counterparts. The peak production of KLH SpAb's showed line and diet effects as depicted in Figure 1. The Control line birds showed peak SpAb production at week 10, one week after the KLH inoculation (irrespective of diet), while the Low line birds showed peak production at week 11 (irrespective of diet), two weeks after the KLH inoculation. Only the High line birds showed diet effects on peak SpAb production, wherein the High line birds fed diet A showed peak production at week 10 and the High line birds fed diet B showed peak production at week 11. Both the High and Low line birds showed differences in SpAb production due to diet during the late phase response to the KLH inoculation.

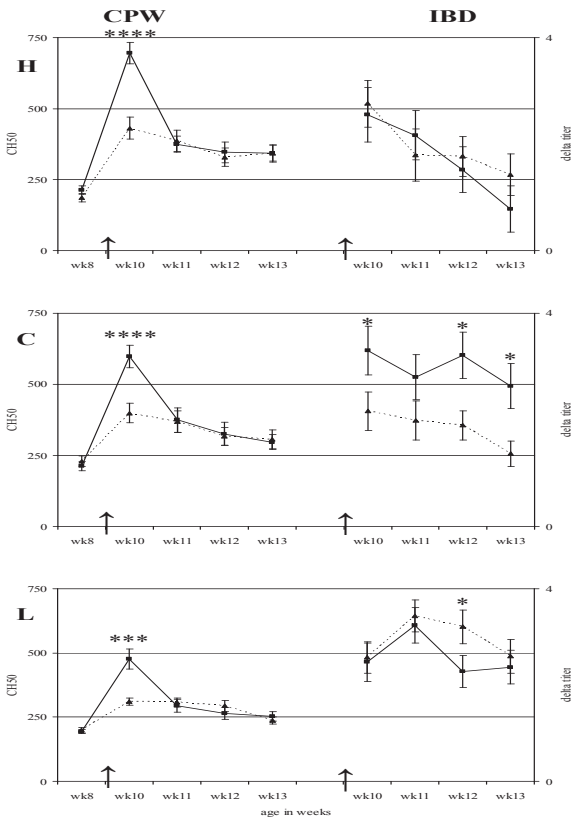

Figure 2. CPW and IBD delta titers in hens of the High (H), Control (C) and Low (L) line on respectively diet A (■, solid line) or diet B (▲, hatched line). Data are mean ± SEM. Group size is n=21-26 per line/diet. ↑ arrow indicates KLH inoculation. * $p<0.05$; *** $p<0.001$; **** $p < 0.0001$. Delta titers were calculated by increase/decrease in absolute titers between the first and all subsequent measurements (e.g. wk13 – wk10, or wk11 – wk10).

Table 2. Measured cellular immune parameters[1] measured one week after the KLH inoculation (wk 10) during the early phase response in High (H), Control (C) and Low (L) line hens.

Line[2]	Diet	WB Control	WB LPS	WB ConA	WB KLH	Mono Control	Mono LPS	PBMC Control	PBMC LPS	PBMC ConA	PBMC KLH
H	A	1006^{a}	2170^{a,x}	31751^{a,b}	1396^{a}	0.53^{a,b}	5.40	1559^{x}	1474^{b}	8298^{a}	2337^{a}
C		779^{b}	2340^{a,x}	30439^{b}	914^{c}	1.24^{a}	5.01	1653	1918^{a}	5453^{b,x}	1868^{b}
L		769^{b,x}	1466^{b}	35964^{a}	1180^{b,x}	0.02^{b}	3.26	1640	1886^{a}	3391^{c}	2001^{a,b}
H	B	974^{a,b}	1094^{b,y}	31318^{a,b}	1465^{a}	0.38	3.80	2027^{y}	1726	6970^{a}	2458^{a}
C		830^{b}	1484^{a,b,y}	26317^{b}	1074^{b}	0.19	5.97	1690	1605	3406^{b,y}	1631^{b}
L		1098^{a,y}	1921a	31728^{a}	1462^{a,y}	0.08	3.87	1939	1743	4139^{b}	2160^{a}
SEM		66.3	233.4	2094	90.9	0.46	0.97	173	152	606	201
Main Effects[3]											
Diet		0.0334 B>A	0.0109 A>B	0.0890 A>B	0.0232 B>A	NS	NS	0.0610 B>A	NS	NS	NS
Line		0.0194 H>L>C	NS	0.0387 L>H>C	<0.0001 H>L>C	NS	NS	NS	NS	<0.0001 H>C>L	0.0071 H>L>C
DxL		0.0188	0.0024	NS	NS	NS	NS	NS	NS	NS	NS

*WB control = LST of whole blood controls; WB LPS = LST of whole blood with B-cell mitogen LPS; WB ConA = LST of whole blood with T-cell mitogen ConA; WB KLH = LST whole blood with antigen KLH; Mono control = monocyte reactivity of RPMI controls; Mono LPS = monocyte reactivity to LPS; PBMC control = LST of PBMC controls; PBMC LPS = LST of PBMC with B-cell mitogen LPS; PBMC Con A = LST of PBMC with T-cell mitogen Con A; PBMC KLH = LST of PBMC with antigen KLH.

[1] Data are group (least square) mean of repeated measures, n=21-26 per line/feed. Within diet contrast between lines with no common superscript differ significantly: a,b,c< 0.05. Statistical difference within line between diets: x,y< 0.05.

[2] Line: H = High line; C = Control line, L = Low line.

[3] Main effects based on Main Effect Means: D = Diet; L = line; DxL = diet by line interaction. NS = no significance, otherwise p value is noted.

Table 3. Measured cellular immune parameters[1] measured four weeks after the KLH inoculation during the late phase response (wk 13) in High (H), Control (C) and Low (L) line hens.

Line[2]	Diet	WB Control	WB LPS	WB ConA	WB KLH	Mono Control	Mono LPS	PBMC Control	PBMC LPS	PBMC ConA	PBMC KLH
H	A	959^{a}	1017^{b}	27375	979^{a}	1.01^{a}	2.65^{b,x}	1954^{a}	2145^{a}	10233^{a}	2407^{a,x}
C		804^{b,x}	989^{b}	28200^{x}	819^{b}	0.66^{a}	2.22^{b}	1226^{b,x}	1425^{b,x}	6213^{b}	1630^{b}
L		987^{a}	1258^{a,x}	27531	990^{a}	0.16^{b,x}	3.95^{a}	1749^{a}	1936^{a}	4198^{b}	1765^{b}
H	B	1016^{a}	1164^{a}	27051^{a,b}	951	0.92^{a}	3.80^{a,y}	1933^{a}	2100^{a}	9032^{a}	1921^{a,y}
C		947^{a,b,y}	971^{b}	22953^{b,y}	898	0.38^{b}	2.79^{b}	1856^{a,y}	1926^{a,b,y}	4249^{b}	1790^{a,b}
L		891^{b}	998^{b,y}	29872^{a}	920	0.96^{a,y}	4.42^{a}	1554^{b}	1655^{b}	3481^{b}	1559^{b}
SEM		50.9	72.1	1817	48.1	0.15	0.41	111.1	96.7	1005	135
Main Effects[3]											
Diet		NS	NS	NS	NS	NS	0.0339 B>A	NS	NS	NS	NS
Line		0.0944 H>L>C	NS	NS	0.0578 H>L>C	0.0070 H>L>C	0.0005 L>H>C	0.0012 H>L>C	0.0039 H>C>L	<0.0001 H>C>L	0.0003 H>C>L
DxL		0.0662	0.0181	NS	NS	0.0012	NS	0.0008	0.0172	NS	0.0628

[*]WB control = LST of whole blood controls; WB LPS = LST of whole blood with B-cell mitogen LPS; WB ConA = LST of whole blood with T-cell mitogen ConA; WB KLH = LST whole blood with antigen KLH; Mono control = monocyte reactivity of RPMI controls; Mono LPS = monocyte reactivity to LPS; PBMC control = LST of PBMC controls; PBMC LPS = LST of PBMC with B-cell mitogen LPS; PBMC Con A = LST of PBMC with T-cell mitogen Con A; PBMC KLH = LST of PBMC with antigen KLH.

[1] Data are group (least square) mean of repeated measures, n=21-26 per line/feed. Within diet contrast between lines with no common superscript differ significantly: a,b< 0.05. Statistical difference within line between diets: x,y< 0.05.

[2]Line: H = High line; C = Control line, L = Low line.

[3]Main effects based on Main Effect Means: D = Diet; L = line; DxL = diet by line interaction. NS = no significance, otherwise p value is noted.

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Humoral immune parameters of innate and specific immunity were measured. APW showed no interaction with diet, although significant line interactions were found (Table 1). CPW on the other hand showed a strong diet effect (p= 0.0286) that was observed the week after the KLH inoculation in all selection lines fed diet A (Figure 2). This effect was greatest in the High line birds followed respectively by the Control and Low line birds fed diet A.

The vaccine specific Ab's to both NCD and IBD showed significant three way interactions between diet, line and time (Table 1). As the delta titers to NCD were still low, the biological relevance of this finding is questionable. On the other hand the IBD SpAb showed significant line-specific differences throughout time (Figure 2), with the greatest differences in the Control line birds. Furthermore, the line characteristics were reversed, with the Low line birds showing the greater production after the KLH inoculation followed by the Control and High line hens. The High line birds showed no diet effects in this parameter, while the Control line birds fed diet A showed significantly higher IBD SpAb production over time when compared with the Control line birds fed diet B and the Low line birds fed diet B at wk 12 showed greater SpAb production when compared with their counterparts, being the week after the KLH inoculation.

The Natural Ab's binding (NAb) either LTA or LPS showed strong three way interactions with diet by line by time (Table 1). Again, all diet effects are line-specific as depicted in Figure 3. LTA binding NAb's also showed a significant diet effect wherein the hens fed diet B showed more LTA binding NAb than the diet A fed birds.

Cellular immune parameters – early phase (wk 10).

All whole blood parameters showed significant diet effects (Table 2). The proliferation of RPMI WB controls was greater in the diet B fed birds (p = 0.0334), wherein the Low line birds showed the greatest difference due to diet. The WB LST with LPS was greater in the diet A fed birds (p = 0.0109), here the High and Control line birds showed significant differences due to diet. The antigen specific LST with KLH showed a significant difference due to diet wherein the diet B fed birds showed greater proliferation to KLH than the diet A fed birds (p = 0.0232). The WB controls and the WB LST with LPS showed significant diet by line interaction.

The NO production from monocytes showed no detectable differences in the early phase response to the KLH inoculation (Table 2).

The RPMI PBMC controls showed a diet effect, wherein the PBMC controls of diet B fed birds were numerically higher than the birds fed diet A (p = 0.0610). This effect was greatest in the High line birds. The PBMC LST with Con

A showed a significant diet effect in the Control line birds, with the diet A fed birds showing greater proliferation to Con A than the diet B fed birds.

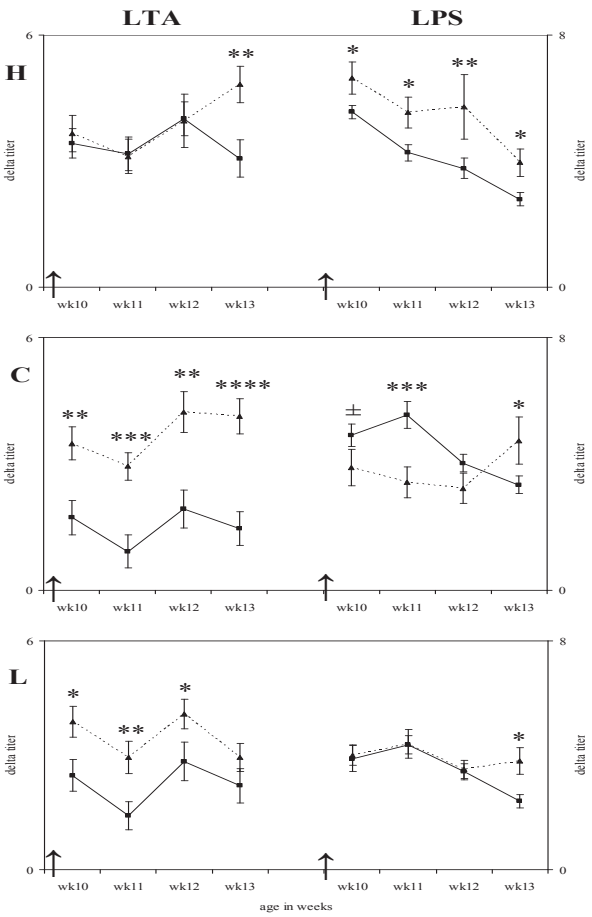

Figure 3. LTA and LPS binding NAb delta titers in hens of the High (H), Control (C) and Low (L) line on respectively diet A (■, solid line) or diet B (▲, hatched line). Data are mean ± SEM. Group size is n=21-26 per line/diet. ↑ arrow indicates KLH inoculation. ± $p<0.1$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p < 0.0001$. Delta titers were calculated by increase/decrease in absolute titers between the first and all subsequent measurements (e.g. wk13 – wk10, or wk11 – wk10).

Cellular immune parameters – late phase (wk 13).

The WB parameters showed no significant diet effects (Table 3), but comparable effects were seen due to diet as were seen in the early phase, but now in a line-specific manner. The Control line birds showed a difference in the RPMI WB controls, with the diet B fed birds showing numerically higher

counts than the diet A fed birds. The WB LST with LPS was different due to diet in the Low line birds, wherein the diet A fed birds showed numerically greater effect than the diet B fed birds. The WB LST with Con A was significantly different in the Control line birds, in which the diet A fed birds showed greater proliferation to Con A than the diet B fed Control line birds.

In contrast with the early phase, the monocytes showed a significant three way interaction between diet, line and time for the medium controls (Table 3). The Low line birds showed the greatest difference in the RPMI medium controls, in which the diet A fed birds showed more NO production than the diet B fed birds. The LPS induced NO production showed a significant diet effect, in which the diet B fed birds showed more NO production than the diet A fed birds ($p = 0.0339$). This effect was greatest in the High line birds.

No diet effects were observed in the PBMC parameters (Table 3), but all PBMC parameters except for the PBMC LST with Con A showed three way interactions between diet, line and time. The RPMI medium controls were significantly different in the Control line birds, in which the diet B fed birds showed numerically greater counts than the diet A fed birds. The Control line birds also showed significant differences in the PBMC LST with LPS, again the diet B fed birds showed greater proliferation to LPS than the diet A fed Control line birds. The High line birds showed a significant difference in Ag specific proliferation to KLH, wherein the diet A fed birds showed greater Ag specific proliferation than the diet B fed birds.

Correlations corticosterone with immune parameters.

All found correlations between measured corticosterone levels and immune parameters are depicted in Table 4. Most of the individual correlations found were weak (0.3 - 0.5). The High line birds fed diet A showed three positive correlations with corticosterone, two of which were with the measured NCD SpAb levels. The High line birds fed diet B showed four correlations with corticosterone, three correlations were negative with humoral parameters (respectively IBD, KLH and LTA) and a positive correlation with monocyte NO production was found.

The Control line birds fed diet A showed moderate to strong correlations with respectively KLH SpAb and LTA NAb's ($r = - 0.61958$, $r = - 0.81439$) at week 11. Thereafter weak positive as well as negative correlations with PBMC parameters were found (respectively the PBMC LST with KLH and PBMC LST with LPS). The Control line birds fed diet B showed the least correlations with corticosterone, only in WB parameters. The WB controls showed a weak positive correlation with corticosterone and the WB LST with KLH showed a weak

Table 4. Pearson correlations (r) between corticosterone levels and immune parameters throughout the observation period in the High (H), Control (C) and Low (L) line hens.

Line[1]	Diet	Wk	APW	CPW	NCD	IBD	KLH	LTA	LPS	WB Control	WB LPS	WB ConA	WB KLH	Mono Control	PBMC Control	PBMC LPS	PBMC ConA	PBMC KLH
H	A	10										0.35545 0.0747						
		11			0.38134 0.0546													
		12			0.35426 0.0758													
H	B	10					-0.44318 0.0342							0.43041 0.0455				
		11						-0.38444 0.0636										
		12				-0.39347 0.0700												
C	A	11					-0.61958 0.0012	-0.81439 <0.0001										
		13														-0.43170 0.0352		0.38002 0.0670
C	B	10								0.42848 0.0594								
		13											-0.47335 0.0261					
L	A	10	0.38661 0.0755			0.46772 0.0282					0.38230 0.0791		-0.42829 0.0467		-0.46842 0.0322			
		11	0.38418 0.7030		0.42278 0.0444	0.57296 0.0043												
		12		0.39286 0.0637														
		13				0.43604 0.0375			0.35691 0.0946									
L	B	10				-0.36914 0.0759											-0.35091 0.0927	
		11						-0.33969 0.0895										
		12		0.59835 0.0012														
		13													0.35780 0.0791			

[1]APW = alternative complement activity; CPW = classical complement activity; NCD = vaccine specific Ab to NCD; IBD = vaccine specific Ab to IBD; LTA = Natural Ab binding to LTA; LPS = Natural Ab binding to LPS; KLH = Specific Ab to KLH; WB control = LST of whole blood controls; WB LPS = LST of whole blood with B-cell mitogen LPS; WB ConA = LST of whole blood with T-cell mitogen ConA; WB KLH = LST whole blood with antigen KLH; Mono control = monocyte reactivity of RPMI controls; Mono LPS = monocyte reactivity to LPS; PBMC control = LST of PBMC controls; PBMC LPS = LST of PBMC with B-cell mitogen LPS; PBMC Con A = LST of PBMC with T-cell mitogen Con A; PBMC KLH = LST of PBMC with antigen KLH.

Chapter VII

negative correlation with corticosterone.

Overall the Low line birds showed the greatest number of correlations with corticosterone, in which the birds fed diet A showed the most of all. The correlations in the Low line birds that were fed diet A were mostly positive with mainly humoral immune parameters. Of the eleven correlations found, only three were with cellular parameters. The Low line birds fed diet B showed positive as well as negative correlations with humoral and cellular parameters. The Low line birds fed diet B also showed significant strong to moderate negative correlations between the KLH SpAb's and IBD SpAb's over time (data not shown; respectively: wk 10, r = - 0.66550; wk 11, r = - 0.73665; wk 12, r = - 0.63102 and wk 13, r = - 0.55640).

Discussion

We studied the effects of minor differences of micronutrients in diets composed of identical raw ingredients from different origin on immune responses in three chicken lines that had been selected in the past for specific primary antibody response to the T-cell dependent antigen SRBC. In a first generation of hens fed these diets we found greatest differences due to diet in cellular parameters of immunity and less effects on humoral parameters (Adriaansen-Tennekes et al, 2009a; 2009b). Here we proposed that a trigger was necessary to make potential differences in humoral immune capacity detectable in the second generation of hens fed these diets.

Interestingly, the corticosterone responses of all the selection lines reflect the phases of the immune response to the KLH inoculation, with line-specific responses. Initially a rise in the corticosterone levels is seen, coinciding with the early phase of the immune response and this is most clear in the Low line birds. At week 11, being two weeks after the KLH inoculation, the immune response has reached the end of the plateau phase and the start of the late phase, with the corticosterone levels in the High line birds starting to rise in this period. The Control line birds showed the least response in corticosterone levels to the KLH inoculation, although the Control line birds fed diet B showed a slight drop in corticosterone at week 11.

The corticosterone response to the KLH inoculation was significantly different in the Low line offspring, wherein the birds fed diet A showed a quick rise two weeks after the inoculation, with the levels normalizing at a slightly higher level thereafter. On the other hand the Low line birds fed diet B showed a delayed, but higher response over time, suggesting altered HPA axis reactivity of the Low line offspring due to the diets. Such altered HPA axis reactivity in offspring after maternal stress has been reported by several groups (Macrì and Würbel, 2006; Love and Williams, 2008). We have previously reported that the mother hens of the Low line chicks in this experiment showed different corticosterone responses to the diets (Adriaansen et al, 2009b). The fact that the Low line mother hens showed a corticosterone response to diet A and that the subsequent generation from these birds showed a blunted corticosterone response to the KLH inoculation strongly suggest that a stress (like) effect has induced the found effects in the offspring of the hens fed diet A from the first generation.

The KLH SpAb reflect the primary Ab response to the inoculation with the neo-Ag KLH. As has already been reported, the primary Ab response itself is difficult to modulate (Boa-Amponsem et al, 1999), possibly based on the

strong genetic regulation of Ab production (Raberg et al, 2003). Sijben et al (2001) showed that slightly different levels of linoleic acid and linolenic acid can delay the time of the peak response to a KLH inoculation from the early phase (5-7 days) to 10-14 days. Interestingly, we found a three way interaction between diet and line over time in the KLH SpAb's, most likely reflecting the delayed peak response that was observed in the High line birds fed diet B as well as the Low line birds irrespective of diet. The measured differences were small questioning the biological relevance of this result.

In this study we used *delta* titers to detect differences in responsiveness of humoral immune parameters to an immunological trigger, the KLH inoculation. Previously, we reported that the diets used in this experiment induced differences in LTA binding NAb in the second generation of selection lines (Adriaansen-Tennekes et al, 2009b) while no other humoral parameters were affected in the second generation. Here, almost all of the used humoral immune parameters measured in this experiment showed significant interactions between the diets and lines over time due to the KLH inoculation, reflecting altered kinetics of these humoral responses. Delay of the peak response was seen for some parameters, mostly in the Low line birds. Some humoral parameters showed clear diet effects with comparable effects in each selection line, albeit in different magnitudes such as CPW (A>B) and LTA binding NAb's (B>A). Other humoral parameters showed more line-specific effects due to the two diets, such as IBD SpAb's, NCD SpAb's and LPS binding NAb's reflecting altered kinetics of these responses. The Low line birds fed diet B showed a strong trade-off between the KLH SpAb's and the vaccine IBD SpAb's during the whole observation period. All humoral parameters were triggered by the KLH inoculation and showed either generalized responses in all lines or line-specific effects due to the diets.

Mashaly et al (1998) reported neuroendocrine regulation of the Ab response. Interestingly, correlations between corticosterone and IBD SpAb's were found in the High and Low lines, while the Control line showed the greatest differences in the responses due to the two diets. The observed diet effects in the Control line birds may be due to the fact that the Control line birds showed no corticosterone response to the KLH inoculation and therefore only partial regulation of IBD SpAb's. The High and Low line birds both fed diet B showed weak negative correlations with corticosterone and IBD SpAb's, while the Low line birds fed diet A showed consistent weak positive correlations over time. All correlations with LTA were negative and a strong negative correlation was found in the Control line hens fed diet A, possibly contributing to the differences found in this line.

The cellular parameters were measured to assess whether they would adequately reflect immunomodulation by diet after an immunological trigger.

Isolated peripheral PMBC's were used as well as WB peripheral lymphocytes to be able to distinguish between *in situ* effects as well as systemic effects. The WB cellular parameters showed the only significant two way interactions with diet and line over time in the early phase of the immune response, being at week 10. Interestingly, control measurements (WB controls and PMBC controls) reflected diet differences in the same manner in all lines (diet B more intrinsically active cells than cells from diet A fed birds). Consistently, the diet B fed birds showed greater antigen specific proliferation to KLH than the diet A fed birds. The Low line birds fed diet A showed several moderate correlations between cellular parameters and corticosterone during this phase of the immune response. During the late phase of the immune reaction at week 13, most two way interactions were found in the isolated PBMC in contrast with the early phase which showed most two way interactions in the WB parameters. Possibly this difference may reflect systemic effects related to e.g. corticosterone levels during the early phase of the immune response, which in the late phase of the response changes to *in situ* adaptation of cellular reactivity.

Most likely the fact that a trigger was necessary to make detection possible of immunomodulation of humoral parameters, relates to the specificity of these parameters. At the end of the plateau phase of the immune response, two weeks after the KLH inoculation there may be the switch from innate (e.g CPW) and cellular immunity to more humoral adaptive immunity as reflected in results of the analysis of the humoral parameters. Humoral parameters are the end result of the activation of the immune system, making it quite possible that adaptation occurs more easily in the cellular compartment of the immune system. Our results suggest that medium control values of cellular parameters, such as WB LST, PBMC LST and monocytes may (in part) reflect such adaptations.

In conclusion, our results confirm the hypothesis that humoral parameters will reflect immunomodulation by diet, but a trigger is necessary to make detection of such effects possible. The cellular parameters clearly reflect immunomodulation by diet, with different effects depending on the phase of the immune response (early phase more systemic (WB) and late phase more intrinsic (PBMC)). The differences in humoral responses are most likely the result of the found differences in the cellular compartment of the immune system. Furthermore, the relevance of using selection lines with differential HPA axis reactivity when detecting immunomodulation by diet is a prerequisite as effects are often found in a line-specific manner. Possibly the line-specific effects are partially the result of differences in HPA axis reactivity, and are therefore under neuroendocrine control of other hormones next to the found

differences in corticosterone. The altered HPA axis reactivity of the Low line birds, strongly suggests epigenetic effects on the HPA axis due to dietary factors. The exact relationships and underlying mechanisms of immunomodulation by diet in selection lines requires further investigation.

Acknowledgements

This study was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality (LNV), the Rabobank and Triodos Bank, the Netherlands.

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CHAPTER

VIII

Line-specific adaptation to diet in mother hens and offspring of chicken with divergent HPA axis reactivity

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Concept

Abstract

Animals with different temperamental traits show different sensitivity for environmental factors. This was found to be related to differences in stress reactivity and therefore differential hypothamo-pituiitary-adrenal (HPA) axis. Mothers are known to prepare their offspring for the environment they will be born into, through (epi)genetic effects. In this study we hypothesized that animals with different traits, may show line-specific sensitivity for the environmental factor diet over two generations of hens. Three selection lines of chicken with differential HPA axis reactivity (high, low and control line) were fed two different diets (differing in protein content), and the offspring were fed the same diet as the mothers. Body weight, egg production, as well as corticosterone levels were measured. In the maternal generation, the main difference found due to diet was differential corticosterone levels in hens with high HPA axis reactivity. Body weight was not affected. Egg production of the maternal hens was affected in the birds with either high or low HPA axis reactivity in differential manners. Maternal hens of the control line showed no effects due to diet. The offspring generation showed line-specific effects due to the two diets, wherein the birds with high HPA axis reactivity showed differential corticosterone responses to a KLH inoculation, suggesting altered HPA axis reactivity due to the two diets in these birds. Offspring hens of the control line showed differential body weight due to the two diets, while the selection line of hens with low HPA axis reactivity showed no apparent effects due to diet in our measured parameters. In conclusion, our results suggest that although animals with high HPA axis reactivity are most environmentally sensitive, over two generations all birds with differential HPA axis reactivity show forms of adaptation to our environmental factor diet, albeit in a line-specific manner.

Introduction

Female birds transfer nutrients as well as antibodies, RNA, and hormones, to their eggs, which are all thought to be important for offspring development (Price, 1998). They represent examples of so-called maternal effects and are thought to have evolved because they translate the environmental conditions experienced by the mother and her partly heritable physiological state into adaptive phenotypic variation of the offspring (Rossiter et al, 1996; Mousseau and Fox 1998a, 1998b).

Animal temperament has been related to different survival and reproduction of the individual. Temperament has been investigated extensively in laboratory settings, wherein terminology such as coping styles, shyness/boldness, reactivity and plasticity etc. are used and are related to either neuroendocrine and/or behavioral traits (Réale et al, 2007). Independent of the selection criteria used, consistently the extremes of selection lines show one major difference: either a hyper reactive hypothamo-pituitary-adrenal (HPA) axis or a hypo reactive HPA axis. Animals with a hypo reactive HPA axis are less responsive to environmental stimuli than animals with a hyper reactive HPA axis, being very reactive to environmental stimuli (Koolhaas, 2008). Interestingly, such traits are consistent over time and individuals representing both traits are both present in a population (Wolf et al, 2007). In the end the fitness consequences of temperament trait variation differ with year and ecological conditions (Réale et al, 2007).

To date, the field of evolutionary ecology has been dominated by the idea that mothers use yolk hormones to adaptively adjust offspring development, a view that assigns control over hormone deposition and its effects on the offspring to the mother (Müller et al, 2007). Whether the transfer to the egg is an active or passive process of the mother is under discussion (Groothuis and Schwabl, 2008). Most studies in avian models use an experimentally induced rise in corticosterone in either the mother and/or eggs (Pike and Petrie, 2006; Saino et al, 2006). When dietary effects are used as environmental factor, this is often investigated with supplementation of (often) exaggerated amounts of e.g. carotenoids (Symonds et al, 2007). Nahashon et al (2007) has shown that small dietary differences in crude protein affect egg production in guinea fowl. The abovementioned shows that egg production is a multi trait factor, regulated by environmental factors, as well as genetic factors.

As temperament is related to environmental sensitivity, we investigated the effects of diet as environmental factor, on mothers and their offspring in hens originating from the 25th generation of chicken selected for their primary antibody (Ab) response to immunization with sheep red blood cells (SRBC). Furthermore,

diet has been related to effects on body weight and egg production (of the mothers) (Cherala et al, 2006; Nahashon et al, 2007). Lastly, bodyweight and egg production are known to be different in selection lines with different HPA axis reactivity (Mashaly et al, 2000). Three lines were used: the High (H) responders which produce high antibody levels to SRBC after immunization, the Low (L) responders which produce low antibody levels to SRBC after immunization and a random Control (C) line from the same parental stock (Parmentier et al., 1994). Previously we have shown that these birds differ in HPA axis reactivity, the Low line birds have a hyper reactive HPA axis and the High line birds show a hypo reactive HPA axis (Adriaansen-Tennekes et al, 2009). Two nutritionally complete diets, differing in protein content were used as experimental environmental factor. The mother hens were fed the experimental diets from the age of 11 weeks until collection of the eggs. Eggs from the first generation were used to form the offspring generation that was fed the same diet as the corresponding mother hen from hatch till the end of the experiment (8 weeks of age). Here we present that diet, as environmental factor, has differential effects on both mothers and the offspring of birds with different traits characteristics.

Materials and Methods

Birds

Mother hens were ISA Brown (Warren) medium heavy layer hens from three lines. The selection lines were selected for 25 generations prior to this experiment for either high or low primary antibody responses at 5-days after intramuscular immunization (without the use of adjuvant) with SRBC at the age of 35-days. The randomly bred third line is the control line, originating from the same parental stock. The group of mother hens consisted of 72 hens evenly divided over the three selection lines, but not immunized with SRBC. Cockerels (n = 21) from all three selection lines were kept as well. The offspring generation consisted of 150 chicks, 50 hens of each line were used, again without immunization with SRBC. All birds were weighed weekly.

All experiments were approved by the Animal Welfare committee of Wageningen University.

Table 1. Feed composition per age group. All numbers reflect the percentage of the ingredient in the total feed. The raw ingredients for diets A and B were from different sources and all supplements were from the same bulk source.

Age group	Starter 0 – 6 weeks	Grower 7 – 17 weeks	Layer from 18 weeks
Raw ingredients			
Maize	20.05	20.06	25.01
Wheat	30.08	26.51	25.23
Barley	5.01	10.03	5.00
Triticale	12.08	0.00	0.00
Soybeans (heated)	0.00	10.21	19.87
Soy flakes	10.18	20.06	0.00
Peas	10.03	10.03	10.00
Supplements			
Potato protein	7.02	0.00	2.50
MonoCalcFos	1.13	0.73	1.01
FX layers premix	1.00	1.00	1.00
Fat (plant origin)	1.50	0.00	0.52
Salt	0.07	0.09	0.06
Chalk	1.65	1.16	7.65
Shells (broken)	0.00	0.00	2.00
$NaCO_3$	0.09	0.08	0.00
Methionine	0.11	0.04	0.15

[1]All numbers reflect the percentage of the ingredient in the total feed. The raw ingredients for diets A and B were from different sources, and all supplements were from the same bulk source.

Housing

The mother birds were kept according to routine procedures for layer hens in individual battery cages. The offspring hens were kept at 6 birds per enriched cage (2.28 m^2) in a floor housing system. For both mother hens and offspring the light regimen was 14 h of light (0500 to 1900), and temperatures were between

16 and 21°C. All mother chicks were vaccinated against Marek's disease (Poulvac, Fort Dodge Animal Health, Vaals, The Netherlands) and infectious bronchitis (IB)(MA 5, Nobilis-Intervet, Boxmeer, The Netherlands) at hatch, and furthermore IB at d 71 (primer, Poulvac), and d 127 (H52, Nobilis); infectious bursal disease (IBD, Gumboro, D78, Nobilis) at d 21; infectious laryngotracheitis (ILT, Nobilis) at d 87; and New Castle Disease (NCD, clone 30, Nobilis) at d 10, 43, 52, and 115 of age. The offspring received vaccinations against Marek's disease and IB at hatch, IBD at d 21 and NCD at d 10, 28 and 40 of age.

Experimental Feed

All mother hens and offspring received diets that were appropriate for their age, being: starter (from hatch to 6 weeks of age), grower (7-17 weeks) and layer diet (from 18 weeks on). For each appropriate age, two identical experimental diets were made only differing in the source of the raw ingredients (wheat, barley, triticale, peas, maize and soy). Each raw ingredient, e.g. wheat A, was used for all the produced experimental diets designated diet A. Diet composition, and macronutrient contents are shown in Tables 1 and 2, respectively. The main difference between diets A and B was protein content (B>A). The mother hens received commercial diets from hatch, a conventional diet from 7.5 weeks of age (specially made as the experimental diets were of a different structure (less coarse) than the commercial diet) and the experimental diets from the age of 11 weeks. The offspring received the experimental diets from hatch to the end of the observation period.

Table 2. Macronutrient content in the experimental feeds[1]

	First Generation				Second Generation			
	Grower		Layer		Starter		Grower	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Energy KJ/kg	14480	14610	14487	14302	14729	14882	15231	15102
Ash content g/kg	51	48	113	114	64	51	55	54
Raw fibre g/kg	43	42	36	40	36	34	39	39
Tot. carbohydrates g/kg	546	539	555	538	624	620	585	574
Crude fat g/kg	61	59	69	64	42	42	62	53
Protein g/kg	173	192	147	164	151	164	176	199
Moisture g/kg	126	120	116	120	119	123	122	120
Chloride g/kg	1.7	2.0	1.3	1.4	2.3	2.1	1.9	2.3

[1]Starter feed was given 0 to 6 wk of age, grower feed from 7 to 17 wk of age, and layer from 18 wk of age on. For all of the A diets, the same batches of raw ingredients were used, as was done for all the B diets.

Blood sampling

Mother hens and offspring were sampled according to a different sampling schedule. The mother hens were sampled four times after the change to the experimental diets, at ages 12, 15, 19 and 33 weeks. When the chicks of offspring group were 9 weeks of age, all animals received an intramuscular injection in the breast muscle with 1 mg of keyhole limpet hemocyanin (KLH) in 1 ml phosphate buffered saline (PBS, pH 7.2) per bird. The offspring were sampled at ages 8, 10, 11, 12 and 13 weeks. Plasma from heparinized blood was collected and stored at -20º C until further analysis.

Plasma corticosterone

Plasma corticosterone was determined using a radioimmunoassay kit, with detection interval 0 – 2000 ng/ml (IDS, Inc., Bolton, UK).

Egg collection

Eggs were collected each day from the start of the laying period (on average at the age of 20 weeks) up to five months thereafter. Prior to and during egg collection for the offspring, mother hens were inseminated with semen from a cockerel that was from the same selection line and fed the same diet. When the mother hens were 31 weeks of age, for two consecutive weeks, eggs were collected to form the offspring group.

Statistical analysis

The differences in all parameters were analyzed by a three-way ANOVA for the effect of diet, line, time and their interactions using the repeated measurement procedure (with a bird nested within diet and line option). The mean differences due to diet and line were tested with Bonferroni's. Differences between the selection lines were determined by multiple comparison of the means. Correlations between parameters were calculated using Pearson's correlation coefficients. All data are presented as mean ± SEM. All analyses were according to SAS procedures (SAS, 2003).

Results

Plasma corticosterone.

A consistent diet by line interaction was found (F = 2.44 (2), p = 0.0950), with a significant diet effect (F = 4.73 (1), p = 0.0333) wherein diet A fed mother hens showed higher corticosterone levels than the diet B fed birds. Corticosterone levels in all the maternal hens showed a significant increase in time (F = 32.35 (3), p < 0.0001). The Low line birds fed diet A showed a significant increase in plasma corticosterone one week after the change to the experimental diets, when

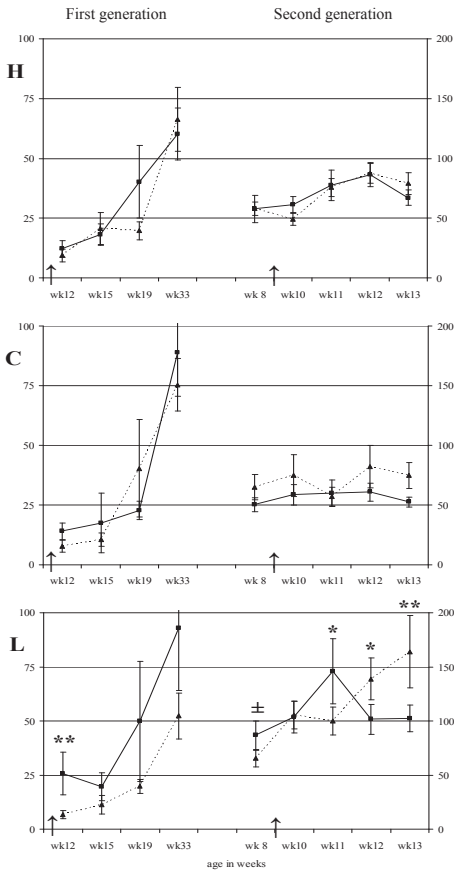

Figure 1. Average corticosterone levels in both generations of hens; High (H), Control (C) and Low (L) line animals on respectively diet A (■, solid line) or diet B (▲, hatched line). Data are mean ± SEM. In the first generation n=11-13 animals per line/diet; second generation group size is n=21-26 per line/diet. ↑ arrow in first generation is start experimental diets; ↑ arrow in second generation indicates KLH inoculation. ± p<0.1; * p<0.05; ** p<0.01.

compared to the Low line birds fed diet B (Figure 1). The corticosterone levels over time were significantly higher in the Low line birds fed diet A, when compared to their counterparts fed diet B ($p = 0.0036$).

In the offspring, a diet by line over time interaction was found ($F = 1.74$ (8), $p = 0.0862$) with a significant diet by time interaction ($F = 3.65$ (4), $p = 0.0061$), as well as an interaction between line and time ($F = 1.96$ (8), $p = 0.0495$). After the inoculation with KLH the corticosterone levels showed line specific responses ($F = 22.24$ (2), $p = < 0.0001$). In all High and Low line offspring the corticosterone levels rise over time (Figure 1). Only the Control line birds showed no corticosterone response to the KLH inoculation. Within the High line birds the corticosterone responses are similar between the two diets. In the Low line birds the greatest differences were found in corticosterone response due to diet, in which the Low line offspring fed diet A showed a quick rise at week 11, and thereafter the return to previous levels. The Low line offspring fed diet B showed a progressive increase in the corticosterone levels with time.

Table 3. Bodyweight[1] and growth per week from High (H), Control (C) and Low (L) line hens on the two experimental diets.

		First generation		Second generation	
Line	Diet	BW (grams)	Growth (g/week)	BW (grams)	Growth (g/week)
H	A	1445^{c}	68.4	419.3^{c}	78.1^{c}
C		1532^{b}	66.2	446.3^{b,x}	81.8^{b,x}
L		1648^{a}	71.7	488.4^{a}	90.4^{a}
H	B	1444^{b}	70.0^{a,b}	425.1^{c}	78.3^{b}
C		1558^{a}	67.1^{b}	525.9^{a,y}	92.8^{a,y}
L		1628^{a}	74.1^{a}	499.7^{b}	90.4^{a}
SEM		30.3	2.45	8.57	1.50
Main effects[2]					
Diet		NS	NS	<0.0001 B>A	0.003 B>A
Line		<0.0001 L>C>H	0.046 L>H>C	<0.0001 L>C>H	<0.0001 L>C>H
DxL		NS	NS	<0.0001	0.0003
Time		<0.0001	<0.0001	<0.0001	<0.0001
DxT		NS	NS	<0.0001	<0.0001
LxT		<0.0001	<0.0001	<0.0001	<0.0001
DxLxT		NS	NS	<0.0001	NS

* Within diet contrast between lines with no common superscript differ significantly: a,b,c < 0.05. Statistical difference within line between diets: x,y < 0.05. Data are group mean: first generation n=11-13 animals per line/diet; second generation n= 21-26 animals per line/diet.

[1]Least squares means ± SEM of the complete observation period after analysis by a repeated measurement procedure after the change to the experimental diets.

[2]Main effects: D = Diet; L = line; DxL = diet by line interaction; T = time; LxT = line by time interaction; DxT = diet by time interaction, DxLxT = diet by line by time interaction. NS = no significance, otherwise p value is noted.

Body weight and growth.

No interaction with diet on bodyweight or growth was found in the maternal birds (Table 3). The bodyweights and growth were significantly different in the selection lines ($F = 20.21$ (2), $p < 0.0001$).

The birth weight of the chicks showed a significant line effect ($F = 8.82$ (2), $p = 0.0002$), with the Control chicks being the heaviest followed respectively by the Low and High line chicks. The chicks from diet B fed hens, showed a significant line dependent difference in birth weight between the High line chicks and the Control and Low line chicks (respectively: High – 31.8 grams, Control – 34.6 grams, Low – 33.8 grams). The chicks from diet A fed birds showed no significant differences in birth weight due to line (respectively High – Control – Low line: 32.6 – 33.9 – 33.2 grams). After one week of feeding the diets, a significant line by diet interaction was found ($F = 9.17$ (2), $p = 0.0002$), as well as two weeks after hatch ($F = 9.17$ (2), $p = 0.0002$) where there was also a significant diet effect ($F = 22.05$ (1), $p < 0.0001$) wherein the chicks fed diet B were heavier.

During the first three weeks of feeding, the High line chicks fed diet A were consistently heavier than the High line chicks fed diet B (at one week after feeding the difference was significant ($p= 0.0034$)), while after four weeks there were no more measureable differences. In the Low line chicks, the chicks fed diet B were significantly heavier than the Low line chicks fed diet A. This difference was apparant up to 6 weeks of receiving the diet, where after there were no more measureable differences.

Bodyweight throughout the whole observation period showed a strong three way interaction between diet and line over time in the offspring ($F = 7.74$ (26), $p < 0.0001$). The offspring showed a strong diet effect on bodyweight ($F = 21.12$ (1), $p < 0.0001$) and with growth ($F = 9.12$ (1), $p = 0.003$), wherein the offspring fed diet B showed more growth and greater bodyweights than the offspring fed diet A. The differences in bodyweight and growth were greatest in the Control line birds (both: $p < 0.0001$).

Table 4. Total numbers of male and female offspring from first generation of High (H), Control (C) and Low (L) line birds on the two experimental diets.

Line	Diet	♂	♀	Total
H	A	37	61	98
C		54	64	118
L		65	66	131
H	B	46	53	99
C		61	68	129
L		33	43	76

* First generation n=11-13 animals per line/diet

Egg data.

The average number of male and female offspring per hen is presented in Table 5. The number of male offspring per hen showed a significant diet by line interaction (F = 4.16 (2), p = 0.0203). The total number of eggs per hen and the average number of female offspring per hen showed diet effects (respectively: F = 3.07 (1), p = 0.0844 and F = 3.35 (1), p = 0.0720), in which the diet A fed mother hens produced more female offspring and more eggs. The Low line

Table 5. Average numbers[1] and percentages of male and female offspring per hen from first generation of High (H), Control (C) and Low (L) line birds on the two experimental diets**.**

Line	Diet	♂	♀	Total	% ♂
H	A	3.7^{b}	6.1^{x}	9.4	$36^{b,x}$
C		$4.5^{a,b}$	5.3	10.2	$45^{a,b}$
L		$5.4^{a,x}$	5.5	11.3^{x}	50^{a}
H	B	$4.6^{a,b}$	$4.2^{b,y}$	7.8	53^{y}
C		5.1^{a}	5.7^{a}	10.4	46
L		$3.3^{b,y}$	$4.3^{a,b}$	7.8^{y}	47
SEM		0.57	0.62	1.1	5.0
Main Effects[2]					
Diet		NS	0.0720 A>B	0.0844 A>B	NS
Line		NS	NS	NS	NS
DxL		0.0203	NS	NS	NS

* Within diet contrast between lines with no common superscript differ significantly: a,b < 0.05. Statistical difference within line between diets: x,y < 0.05. Data are group mean: first generation n=11-13 animals per line/feed.

[1]Least squares means ± SEM of the complete observation period after analysis by a repeated measurement procedure after the change to the experimental diets.

[2]Main effects: D = Diet; L = line; DxL = diet by line interaction. NS = no significance, otherwise p value is noted.

birds showed the greatest effect on number of eggs per hen, wherein the diet A fed birds produced more eggs than the diet B fed birds (p = 0.0324). The greater number of female offspring was significantly higher in the High line birds fed diet A, when compared with the High line birds fed diet B (p = 0.0379). The percentage of male offspring was significantly lower in the High line birds that were fed diet A, when compared to the High line birds fed diet B (p = 0.0222).

Discussion

In this study we aimed to investigate if birds with different traits are differently affected by an environmental factor, and how the offspring of these birds are affected, due to maternal and/or direct effects of the same environmental factor. We used divergently selected chicken with High and Low antibody production and different HPA axis reactivity, as well as the randomly bred control line. As environmental factor we used two nutritionally complete but slightly different diets.

Each selection line of hens showed different effects on different parameters in the mother hens and/or offspring. The mother hens showed no effects due to diet on bodyweight or growth. The measured corticosterone levels of the Low line mother hens fed diet A were significantly elevated when compared with the Low line mother hens fed diet B, suggesting that diet A has induced a stress like response. The corticosterone response to the KLH inoculation in the offspring was significantly different in the Low line chicks, the birds fed diet A showed a quick rise two weeks after the inoculation, with the levels normalizing thereafter. On the other hand the Low line offspring fed diet B showed a delayed, but higher response over time, suggesting altered HPA axis reactivity due to the diets. Such altered HPA axis reactivity in offspring after maternal stress has been reported by several groups (Macrì and Würbel, 2006; Love and Williams, 2008).

The differences found in the High line birds fed the two diets were at the level of the eggs: the percentage of male offspring laid per hen and a trend in the number of eggs laid. The Low line mother hens showed the same difference in number of eggs produced per hen, as was seen in the High line birds, wherein the diet A fed hens produced more eggs than the birds fed diet B. Differences in percentage of male offspring is often suggested to be the result of corticosterone levels in the hen (Love and Williams, 2008). Also the number of eggs produced have been related to corticosterone levels, showing negative (Satterlee et al, 2007) as well as positive correlations (Lowe and Garwood, 1981). However, differences in corticosterone do not explain the results in the current experiment. The corticosterone levels did not differ between the diet A or diet B fed birds of the High line animals, and we found no correlations between the measured levels of corticosterone and these egg parameters. This suggests that other factors than only corticosterone influence egg production and sex ratio. Indeed, this possibility has been proposed by other groups as well (Badyaev et al, 2006; Shultz and Kitaysky, 2008). Next to suggested effects by corticosterone, dietary differences such as the amount of fat, carbohydrates and/or protein in the diet are known to affect number of male offspring as well as the total number of eggs laid per hen

(Grobsa et al, 1999; Safaa et al, 2008; Reynolds et al, 2004; Roberts et al, 2007). Such effects have even been detected with only very small differences in the amounts of crude protein used (Nahashon et al, 2007). The small dietary differences between the used diets here (diet B contained more protein and diet A more fat) suggests that mainly the difference in fat content has affected the number of eggs produced (more fat in diet A, greater numbers of eggs per hen). The difference in sex ratio found in the High line birds fed diet A is most likely the result of several factors as suggested by Badyaev et al (2006).

The Control line offspring showed a difference in body weight due to the two diets, in which the diet B fed birds showed the greater body weights when compared with the diet A fed Control line hens. Lower body weight of offspring has been reported due to early developmental stress in mother Zebra finches (Naguib and Gil, 2005). In rats much work has been done on protein differences in diet on offspring showing that mothers fed the high protein diets, had offspring with greater body weight (da Silva Faria et al., 2004; Cherala et al., 2006). Gorman and Nager (2004) showed increased body weight of offspring from protein supplemented Zebra finches. The isoflavone, genistein from soy, has also been shown to have significant effects on the body weights of offspring, also affecting coat color, through epigenetic effects when fed to pregnant mice (Dolinoy et al., 2007). All the aforementioned factors may have played a role in the found effects of our experiment.

The fact that the Low line birds of the first generation showed a corticosterone response to diet A and that the offspring of these birds showed a blunted corticosterone response to the KLH inoculation strongly suggest that a stress (like) effect has induced the found effects in the offspring of the hens fed diet A. The different selection lines with different Ab production characteristics and different HPA axis reactivity showed different adaptation in the offspring to diet A. The High line birds showed a difference in sex ratio of the offspring as well as more eggs per hen (Love and Williams, 2008; Lowe and Garwood, 1981). The Control line birds showed an altered body weight in the offspring which is in line with the results from Naguib and Gil (2005). And the offspring of Low line birds fed diet A showed a blunted corticosterone response to the KLH inoculation, coinciding with altered HPA axis reactivity of offspring due to maternal stress (Macrì and Würbel, 2006; Love and Williams, 2008). There are several reasons that explain the lack of corticosterone differences in the Control and High line birds. First, our sampling schedule may have missed a rise in corticosterone levels of the High and Control line birds as their response is characterized by a rapid increases, with quick normalization of the corticosterone response (Adriaansen-Tennekes et al, 2009). Cyr and Romero (2007) showed that chronic stress dose

not necessarily affect corticosterone levels in free living birds, but effects can still be detected. Furthermore, the dietary induced corticosterone response in the Low line birds may reflect the greater environmental sensitivity of these birds. Lastly, in the High and Control line birds a different mechanism than corticosterone may have induced the found effects, as these birds are more intrinsically regulated (Koolhaas, 2008).

Our data suggest that diet A may be more environmentally challenging than diet B. Furthermore, diet A induced a corticosterone response that seemed sufficient enough to induce altered HPA axis reactivity in the subsequent generation, suggesting epigenetic regulation of the HPA axis by diet. The selection lines proved to be useful in finding different modes of adaptation to the environmental factor diet. Differences in adaptation have been found previously by using selection lines (Yang et al., 2000; Satterlee et al., 2008). The different modes of adaptation found in these selection lines may well be a factor contributing to the diversity of results when evolutionary, ecological and environmental factors are being investigated.

Acknowledgements

This study was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality (LNV), the Rabobank and Triodos Bank, the Netherlands.

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CHAPTER

IX

General discussion

Multidisciplinary approach

The work presented in this thesis shows that investigating underlying mechanisms of dietary immunomodulation requires a multidisciplinary approach of several systems: the neuroendocrine system, the immune system, genetic background, diet composition, as well as the GALT and microflora in the gut. In this thesis the first three systems were investigated and the role of the GALT and microflora will be elaborated on further in this discussion. Such multidisciplinary relationships have already been described for several autoimmune diseases (Chapter 2), as well as obesity and type-2 diabetes (Hotamisligil and Erbay, 2008), wherein a role was found for the immune system as well as the neuroendocrine system and microflora composition (Bastard et al, 2006; Pasquali et al, 1996; Macia et al, 2006; Serino et al, 2009).

Diet Characterization

The diets used in this study showed only few differences that were consistently present throughout all of the diets prepared for the birds over the two generations. When analyzing the modulatory effects of diets on the neuroendocrine system and immune system it is of importance to realize that 1) possibly the small individual differences found alone are almost meaningless, but in combination with each other do cause observable effects and 2) nutrients presented in whole food sources are more bioavailable than when given in supplemented forms. The first assumption is based on the finding that e.g. low contaminations of LPS or Pb alone have no effect on the immune system, but in combination at the same low doses, the two seem to show synergistic effects causing effects on the functioning of the immune system (Cheng et al, 2006). Furthermore, whole foods as source of e.g. vitamins or other nutritional factors seem to be more bioavailable and more biologically effective than when given in supplemented form only. This has been shown for lycopene from tomatoes when compared with lycopene supplementation only, but also vitamin A, calcium and e.g. olive oil (Basu and Imrhan, 2006; McDaniel et al, 2007; Napoli et al, 2007; Pérez-Jiménez et al, 2007). It was suggested that possibly other components in the whole foods enhance the bioavailability of the individual components in a synergistic way (Woodside et al, 2005; Jacobs and Tapsell, 2007). When attempting to make a generalized characterization of diet A used in this experiment, the following components are most consistently present: isoflavones, more microbes, more cartenoids and more vitamin E (precursors). Especially the first three are all known

to be strong immune stimulatory components, making diet A a more immune stimulatory diet (Hartley et al, 2003; Cook et al, 2006; O'Brien and Dawson, 2008; Puthpongsiriporn et al, 2001; Yang et al, 2000). Diet B on the other hand contains more tryptophan, vitamin C, protein and more bioavailable amino acids. These components are known to have effects on stress reactivity, all being stress reducing working via the HPA-axis (Peeters et al, 2006; Koopmans et al, 2006; Jones et al, 1999; Markus, 2008).

Neuroendocrine parameters

Chapter 2 clearly showed that although the HPA axis response to CRH was identical, the behaviouraly defined coping styles reflect differences in disease susceptibility. The use of the selection lines was paramount for the investigation of the neuroendocrine system during immunomodulation by diet. The selection lines showed differential HPA axis reactivity to mild stress, as well as line-specific effects on immune parameters due to stress (Chapters 3 and 4, respectively). Interestingly, the Control line showed the least HPA axis response and no informative effects on the measured immune parameters due to the applied mild stress. Furthermore, diet induced a plasma corticosterone response in the birds with high HPA axis reactivity, which is in line with the concept that animals with high HPA axis reactivity are more environmentally sensitive (Chapters 5 and 6). When the offspring generation of hens was triggered by KLH, again, differences in neuroendocrine regulation were found in the hens with high HPA axis reactivity (Chapter 7). It remains to be determined if the different corticosterone responses of the Low line birds of the offspring generation were due to epigenetic factors and/or due to effects of the diets directly (Chapter 8). The work presented here shows that diet can indeed influence the neuroendocrine system in animals with high HPA axis reactivity.

Immune parameters

The effects of stress were reflected on different immune parameters in the three selection lines used (Chapter 3). The line-specific differences are most likely related to the different neuroendocrine regulation in these selection lines, as well as the accompanying differences in hormone receptor sensitivity (Rohleder et al, 2003). Most hormones are, to a more or lesser extent, related to many

immune diseases, and diseases such as metabolic syndrome, obesitas, depression and diabetes also show immune dysregulation (Guijarro et al, 2006; Elenkov et al, 2006). Diet mostly affected the cellular compartment of the immune system, reflected in the altered in situ reactivity of monocytes, as well as the proliferative capacity of lymphocytes (Chapter 6) in the first generation of birds. This suggests that diet mainly alters the in situ capacity of immune cells in a relatively short term fashion. The selection lines showed a slight divergence, with the Low line birds showing the greatest effect in the lymphocyte proliferation and the High line birds showing the greatest effect on monocyte reactivity. Both these observations underscore that effects on the immune system are relatively upstream, early in the immune response. As the Low line birds of the first generation also showed altered Ab levels to standard vaccines, a clear downstream effect due to diets was found as well. Possibly the altered vaccine Ab levels were the result of the greater rise in corticosterone, as stress has been proposed to act as an adjuvant to immunization (Viswanathan et al, 2005). As the Low line birds are more environmentally sensitive, it is likely that the monocyte is (one of the primary) immune cell(s) used to sense environmental changes and to avoid overreactions to environmental cues a tight neuroendocrine regulation is necessary, as was reflected in the corticosterone levels.

The neuroendocrine approach to detect potential downstream effects of diet on the immune system (Chapter 7), was most successful in the offspring generation. The response to the immune trigger, KLH, induced clear differences in kinetics of the immune responses on both the cellular level as well as the humoral level. At the humoral level innate parameters were mostly affected, e.g. CPW in the High line and LTA NAb's in the Low line, with the Control line showing both effects. Early in the immune response to KLH mostly systemic effects were seen in the measured whole blood cellular parameters, while at the end of the KLH response an altered immune status of the birds was seen in NAb's as well as cellular parameters. Again, correlations with corticosterone levels were found, suggesting partial regulation by the neuroendocrine system.

The use of two generations revealed differences over generations. Results with the first generation suggested that mainly the Low line birds were sensitive to dietary immunomodulation, while in the offspring generation all lines appeared to be sensitive to immunomodulation by diet. All the selection lines of the offspring generation showed a form a adaptation to the used diets as is presented in Chapter 8. Whether the found effects were the result of epigenetics and/or the fact that the offspring generation received the diets from hatch needs to be further established.

Individual differences

Koolhaas (1999), Ellenbroek and Cools (2002) and Øverli (2007) have reviewed coping style characteristics for other species than rodents alone and here we would like to elaborate on this for chicken. As stated previously by Koolhaas (1999) feather pecking (FP) behaviour can be interpreted as part of the characteristics in selection lines, where proactive animals are more prone to show feather pecking than reactive birds. Several chicken lines have been established on basis of the tendency to show feather pecking behaviour and when observations in behaviour and e.g. neuroendocrine system are further examined, the selection lines showing high FP show many characteristics of the proactive strategy. The selection lines showing low FP behaviour in turn show many characteristics of the reactive strategy (Korte et al, 1997; Jensen et al, 2005). In chicken FP has also extensively been examined through QTL research and recently it was found that the main gene contributing to this behavior is PMEL17 (Keeling et al, 2004; Nätt et al, 2007). This gene was found to be related to eumalanin synthesis which in turn is needed in the metabolic pathway for dopamine and catecholamines, two systems that are distinctly different in the proactive and reactive strategies (Koolhaas, 2007; Ellenbroek and Cools, 2002). Another interesting feature of PMEL17 is that it is also related to feather color explaining that white chickens are the birds showing the most feather pecking behaviour while the "coloured" birds are the birds that are most pecked at and show the least FP behaviour (Keeling et al, 2004). Possibly in chicken, feather color directly relates to the strategy the birds have, with white birds being the more proactive and the colored birds being the more reactive (the darker the feathers the more reactive). Although highly speculative it is tempting to speculate that white chicken are the birds that are most rigid and therefore function best under stable conditions and in turn the darker colored birds are most flexible and therefore function best under changing conditions. Albentosa et al (2003) shows that this may hold true within selection lines of one parental strain, but between different strains of chicken with different colors the distinction isn't as clear cut.

The selection lines used in this thesis also reflect the above mentioned two strategies, wherein the high line birds showed the proactive strategy and the low line birds coincide with the reactive strategy (Chapter 3 and 4). Interestingly, the immune parameters in chicken selection lines that were selected for FP behavior show comparable characteristics as observed in the selection lines used here, with the birds showing most FP have strong humoral (antibody) reactions and the birds showing least FP seem to have stronger cellular regulation as is seen in the present Low line birds (Buitenhuis et al, 2004, 2006). Other selection lines that

have been selected on immune parameters seem to show comparable dichotomy, making a comparable genetic basis likely (Dennis et al, 2006). Also profound differences between other selection lines and the present selection lines used here were found (Minozzi et al, 2008). This may partially be due to the number of generations wherein the selection criteria have been used. The selection lines is this experiment have been selected over 25 generations, most FP selection lines are still in the first five generations of selection, possibly making the phenotypes less defined as is seen with the present high and low antibody forming lines. Further differences are caused by e.g. sex (male-female), used stress paradigm: often immune parameters are determined after specific stress and each form of stress may cause a different response, age (where young animals may show different responses when e.g. in an imprinting period and adult responses may be more rigid), and last but not least: the selection procedure in itself may skew further (neuroendocrine and immunological) responses more than we realize to date (as it is part of the environmental stimuli that help form the individual) (Ellenbroek, 2002).

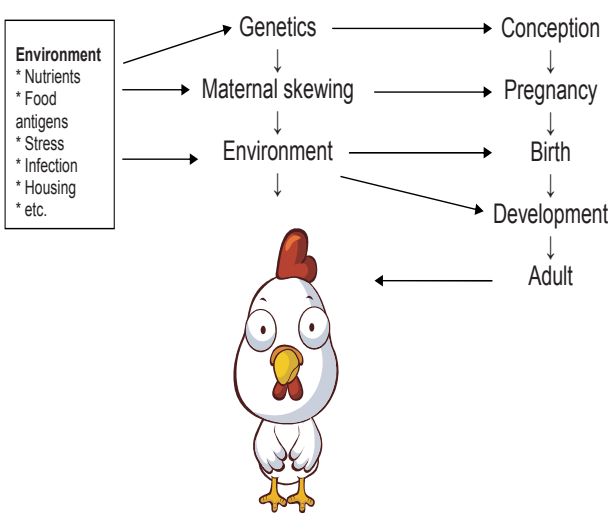

Figure 1. Proposed factors contributing to the adult phenotype.

Disease susceptibility is therefore a complex interplay between several systems but also with sensitive periods wherein sometimes effects are obtained that are irreversible. Genetic makeup plays the first role in determining the primary dispositions, but through maternal skewing and subsequently through environmental factors much fine tuning is achieved to adapt each individual as much as possible to the current environmental conditions (Figure 1). By definition this process cannot be beneficial to all the organisms in the population. Within a population the primary function of the individual is to assist in the survival of the population, this can be achieved in many different ways. Immunologically speaking this means that there must be sufficient immunological diversity within the population to ensure that during an epidemic a part of the population will survive.

The above illustrates the complexity of interventions aiming to enhance immunity. With the progress that is being made, in time individual therapies and/or diets will become available. Can this also be achieved for our farm animals? It seems unlikely, as already there has been strong genetic skewing towards production parameters only at the cost of other characteristics, including capacity for immune defense. The normal genetic variance that was present in wild type, is long gone (Coe et al, 2008).

Hygiene hypothesis: microbes, microflora or both?

To date there are two interpretations of the hygiene hypothesis wherein one interpretation states that immune stimulation with microbes early in life is necessary for recognition of innocuous antigens later on (based on the Th1/Th2 dichotomy; Von Mutius, 2007; Garn and Renz, 2007), while the other interpretation states that the use of antibiotics and dietary differences cause perturbations in gastrointestinal microbiota composition which in their turn have an effect on immunological tolerance (Noverr and Huffnagle, 2005). Here we would like to assume that the two interpretations may both hold true (Vercelli, 2006), but with regard to the results of our experiments we would like to discuss our results in light of the microbiota composition. In this theory the stage of dentritic cell (DC) maturation determines the induction of either Th1, Th2 or regulatory T cells. The maturation of the DC is induced by several microbes: probiotic bacteria induce DCr (regulatory DC) which in turn induce Treg, LPS and yeast induce DC1 which in turn induces Th1 and Hyphae and Helminths induce maturation of Dc into DC2 which in turn induce Th2 subsets (reviewed by Noverr and Huffnagle; 2005). The Treg pool that is induced by DCr by symbiotic and

Table 1. Within comparison and three way interactions between diet, line and feed extract in whole blood cultures1 from High (H), Control (C) and Low (L) line birds with a suboptimal concentration ConA. The cultures with extracts (E) from the diets (D) were done eight weeks after the start of the experimental feeds in the first generation and three weeks after the KLH inoculation in the second generation of birds.

		First Generation			Second Generation		
Line[2]	D - E	1:10000	1:1000	1:100	1:10000	1:1000	1:100
H	A-A 1	1.05	1.38	1.67	1.30^{z}	1.44^{x}	1.21
	A-B 2	1.20	1.49	1.21	1.24^{z}	1.38^{y}	1.17
	B-A 3	1.14^{a}	1.54	1.95	0.93	1.16	1.25
	B-B 4	1.36	1.68	1.58	0.99	1.13	1.24
C	A-A	0.93	1.13	1.60	1.32^{z,c}	1.53^{z,c}	1.47^{x,c}
	A-B	1.06	1.34	1.59	0.97	0.93^{x}	0.92
	B-A	1.12	1.13	1.33	0.90	1.04	1.21
	B-B	1.22	1.35	1.30	1.03	1.12	1.11
L	A-A	1.12	1.31	1.37	1.01^{a}	1.21	0.99
	A-B	1.23	1.55	1.01	1.18^{y}	0.94	0.79
	B-A	1.22	1.51	1.28	1.03	0.98	0.78
	B-B	1.22	1.39	0.81	0.98	0.90	0.73
SEM		0.07	0.12	0.20	0.05	0.07	0.08
Main effects[3]							
D		0.0066 B>A	NS	NS	<0.0001 A>B	<0.0001 A>B	NS
E		0.0046 B>A	0.0632 B>A	0.0160 A>B	NS	0.0005 A>B	0.0007 A>B
L		0.0464 C>H>L	0.0040 H>L>C	0.0029 H>C>L	NS	<0.0001 H>C>L	<0.0001 H>C>L
DxE		NS	NS	NS	0.0334	0.0010	0.0247
DxL		NS	NS	NS	0.0087	NS	NS
ExL		NS	NS	NS	0.0642	NS	0.0337
DxExL		NS	NS	NS	<0.0001	0.0029	NS

* Significant contrast within selection line and diet are (1vs2 or 3vs4): a: p< 0.05; b: p< 0.01; c: p< 0.001
* Significant contrast within selection line and extract are (1vs3 or 2vs4): x: p< 0.05; y: p< 0.01; z: p< 0.001
[1]Data are group mean: first generation n=11-13 animals per line/feed; second generation n=21-26 per line/feed.
[2]Line: H = High line; C = Control line, L = Low line
[3]Main effects: D = Diet; E = extract; L = line; DxE = diet by extract interaction; DxL = diet by line interaction; ExL = extract by line interaction; DxExL = diet by extract by line interaction.

pathogenic bacteria are necessary for host immune homeostasis and enhance the immune threshold required for immune activation and the induction of the immune response (Hrncir et al, 2008; Belkaid, 2007). Animals with no Tregs show enhanced T cell responses, something that was observed in the birds fed diet

A (proliferation to ConA, Chapter 6). Here we would like to postulate that the birds fed diet A may have a poorly developed or essentially different microflora composition and therefore showed more enhanced T cell responses. The birds fed diet B may have a much more rich microflora and therefore these birds may have developed more Tregs with the accompanying enhanced immune threshold. Using extracts of the diets in in vitro proliferation assays (Table 1) in the offspring generation, an increased proliferation of whole blood cells was found in birds fed diet A with both extracts as well as enhanced proliferation with the extract from diet A suggesting that no tolerance was induced in the diet A fed birds. The immune responses of all selection lines to diet A suggest a hyperresponsive state to the KLH inoculation, while the response of the diet B fed birds suggest a more regulated and balanced immune response.

Currently, Th17 cells are proposed to be the main defense to extracellular pathogens such as fungi and/or bacteria (Afzali et al, 2007; Stockinger and Veldhoen, 2007). Diet A showed consistently higher numbers of microorganisms in the microbiological assays, and in addition also increased CFU derived from fungi. Possibly the diet A fed birds have more Th17 cells due to this when compared with the B fed birds. In general this would mean that the A fed birds have a greater Th17 pool and the B fed birds would have a greater Treg pool. This would be in line with the fact that all offspring hens, irrespective of selection line, were affected by the diets, most likely due to the fact that the offspring generation received the diets from hatch, therefore having a greater effect on potential Treg development. This would need to be further examined in the future.

Immunomodulation by diet: how?

The main chain of reactions in a typical immune response is via innate immune cells that activate antigen presenting cells (APC e.g. DC or Mφ) which subsequently activate cells of adaptive immunity (see Figure 1, introduction). The results in this thesis indicate that the (peripheral) monocyte seems a key player in inducing subsequent immunomodulation by the diets. Each week after a diet change a great increase was found in the in situ reactivity of the monocytes, but in the measurement 4 weeks after the diet change absolutely no difference in in situ monocyte reactivity was found. These results strongly suggest the central role for monocytes (and other APC) in the subsequently found effects. The subsequent effects of these reactive monocytes may have resulted in different maturation of the APC with possible skewing of the immune responses towards Th1 or Th2 depending on the selection line and/or inducing Th17 or Treg differentiation.

The Low line birds showed a clear corticosterone response to diet A and due to our sampling schedule it cannot be ruled out that there was also a corticosterone response to diet B as well. This corticosterone response suggests activation of the stress axis, which is differentially regulated in these birds (Chapters 3 and 4). During stress conditions, not only corticosterone levels are elevated, but also other hormones such as ACTH may be activated depending on the selection line. Interestingly, as mentioned in the introduction, monocytes/macrophages have been shown to posses almost all hormone receptors, possibly therefore acting as bridge between the hormone response and the immune response.

The gut may coordinate both the effect on monocytes as well as the measured corticosteron response. Part of the immune modulation may have occurred via modulation of the gut microflora as previously proposed in the hygiene hypothesis by regulation of Treg and/or Th17 cells.

Allostasis vs. homeostasis

As previously stated, the road to adulthood can skew the neuroendocrine system and/or the immune system, possibly resulting in increased or decreased susceptibility for disease. The dog can be used as easy example for this phenomenon: each dog owner is told to socialize their animal when the dog is 8 – 12 weeks old. This period is called the imprinting period. Everything the dog will encounter in this period will be accepted by the dog as being normal and will thereafter not provoke a stress reaction. It is temping to suggest that the neuroendocrine system is "calibrated" in this period. For the immune system, a comparable imprinting period has been suggested (Spencer et al, 2006; Hasselquist and Nilsson, 2008). The greater number of encounters during this period, the greater the adaptability of the dog later in life. This may hold true for the immune system as well. The allostasis model fits in this concept of imprinting, as triggering of the systems is necessary for adequate adaptability, without inducing enhanced susceptibility for disease (Horton, 2005; Spencer et al, 2006). Most laboratory animals, and production animals are held in scarce housing conditions with little environmental triggers during these imprinting periods, while enriched housing and neonatal handling have been shown to reduce stress reactivity later in life (Francis et al, 2002; Benarova-Milshtein et al, 2004; Panagiotaropoulos et al, 2004). Different housing systems also influence immunocompetence of animals (Millet et al, 2005; De Groot et al, 2000).

As there are strong individual differences in animals and humans, adaptation processes may differ depending on the coping style (Degen et al, 2004).

For example birds with a reactive coping style, may require more environmental cues for adequate adaptation of the HPA axis, while proactive copers may require more cues that trigger intrinsic mechanisms. Chapter 8 shows that adaptation to the two diets is different in all the selection lines and is diet dependent. The adaptive effects found in the diet A animals have previously been characterized as adaptations that occur under challenging environmental conditions (Saino et al, 2005; Mitchell and Read, 2005; Taborsky, 2006; Dubiec et al, 2006).

Classically corticosterone levels are used as a marker for stress. If imprinting periods are necessary for the calibration of bodily systems, stress, or better, corticosterone may actually be a requirement in this adaptation process, the magnitude being coping style dependent. This in turn would imply that the classical definition of stress and its markers may need reassessing, wherein paradigms representing mild stress are "normal" and cause adaptation of the individual and reflect disease susceptibility, while severe stress remains the important factor in disease induction. In this setting, the amplitude of a response after re-exposure may reflect the amount of adaptation to e.g. environmental factors.

Multidisciplinary approach revisited

Using concepts such as coping styles for an integrated approach to e.g. disease susceptibility, can be a useful tool in understanding the individual differences in the results found here. Putting previous differences described for these selection lines that were also used in this thesis in this context will further elucidate this.

Previously described immune parameters in the used selection lines showed that the Low line birds have more γδT-cells (Parmentier et al, 1995). Interestingly, these cells have been proposed to be related to environmental cues which coincides with the concept of coping styles and the proposition that the Low line birds are reactive copers, and more environmentally sensitive (Percival et al, 2008). Furthermore, quail differing in coping styles show no differences in HPA axis reactivity when they are confronted with e.g. a novel object (Richard et al, 2008); a comparable effect is observed in the immune response in the selection lines of chicken used in the experiments of this thesis. E.g when immunization is performed in the presence of Complete Freund's Adjuvant (CFA), line differences in Ab production are no longer present (Kreukniet et al, 1992). Again, reactivity is the main distinction between coping styles, not baseline, stress free measurements. The selection lines also display a difference in susceptibility for different infections, as was seen with contrasting reactivity to KLH and *Myco-*

bacterium butyricum (Buitenhuis et al, 2004; Minozzi et al, 2008). It remains interesting that the generalized concept that animals from different coping styles are either more environmentally regulated or intrinsically regulated is also reflected in disease susceptibility.

On the other hand, all selection lines of the offspring generation showed a form of adaptation to diet as a prototypical environmental factor. This directly illustrates that although the concept of coping styles is very useful in understanding results without generation effects, this concept is much more complex when investigating effects over different generations. Here an attempt will be made to put the observed diet related effects in the offspring generation in the context of coping styles.

The High line birds fed diet A showed an altered sex ratio in the offspring with more females and less males. As stated in Chapter 8, such sex ratio effects are often attributed to differences in corticosterone levels, but this was not found in our results. Although mechanisms have been proposed, other than corticosterone and/or protein or fat levels in the diet, yet another possibility may be maternal stress prior to egg laying as was observed with supplemented protein before egg laying in zebra finches (Gorman and Nager, 2004). Corticosterone levels found in the High line hens fed diet A, prior to the egg collection period, showed significant strong correlations with the number and percentage of male offspring (respectively: $r = 0.72767$, $r = 0.72481$). Furthermore, male birds from stressed mothers have been found to show better flight (Chin et al, 2008). This may suggest that although less male offspring are produced, the survival chances of these birds is greater due to better flight, supporting the concept that every advantage has its disadvantage and vice versa. Furthermore, this form of adaptation ensures that animals with less capacity to adapt to the environment, will have enhanced survival chances in a form that is most suitable to its coping style.

The Low line birds fed diet A produced more eggs. In invertebrates, greater numbers of offspring are often produced in challenging environmental conditions to enhance the survival chances of offspring (Marshall et al, 2008; Brown and Shine, 2009). Possibly animals with high HPA axis reactivity, such as the Low line hens, use such a strategy as well. In the light of coping styles, animals that are more environmentally sensitive, have a greater chance that the adaptation to a challenging environment may not be successful, requiring greater offspring numbers to enhance the chance that a few will succeed.

Although the generalized assumption is that animals with greater immunocompetence, such as the High line birds in this thesis are more healthy due to their greater specific immune reactivity, these birds show little to no flexibility as was observed in the results presented in this thesis as well as work done by

Hangalapura et al (2003, 2004, 2005). Such animals present with very ridged, routine like behavior as well as low adaptability to the environmental conditions present. Furthermore, upon re-exposure to the same antigen, previous line differences are no longer present (Boa-Amponsem et al, 1999). On the other hand, reactive copers, such as the Low line birds, show much more flexibility as well as adaptability in the neuroendocrine system as well as the immune system. Therefore the notion that the higher the response, the better/more healthy is oversimplified as it is the environmental conditions that determine which is best (Réale et al, 2007; Kawasaki et al, 2008).

In conclusion, the overall results in this thesis show that immunomodulation by diet is possible and is regulated differently in the various selection lines used, coinciding with the concept that animals with different coping styles show different sensitivity to environmental factors. The Low line hens with low Ab production and high HPA axis reactivity show strong regulation of monocytes by corticosterone resulting in lower intrinsic immune responsiveness. The High line birds with high Ab production and low HPA axis reactivity show greatest effects on monocytes, most likely due to the lesser regulation by corticosterone. It appears that the difference between these two selection lines is strongly based on genetic factors that distinguish these birds as was also seen in the work done Biozzi et al (1975), wherein the selection procedure has created birds with either strong adaptive immunity, as was seen in the High line hens and on the other hand the Low line birds with stronger innate immunity as was also reported by Araujo et al (2000). The Control line birds show the least reactivity in corticosterone to our diets, with intermediate effects on monocyte reactivity in the first generation, but the greatest effects on monocyte reactivity in the second generation in which a corticosterone response to the KLH inoculation was absent, supporting the hypothesis that monocytes (APC) are paramount in the induction of immunomodulation by diet. All further downstream effects are most likely the result of interactions between hormone responses with cells of the immune system.

Future work

Although the extremes of selection lines, as the lines used here, are paramount in investigating underlying mechanisms in disease and stress, these extremes represent only 10 – 20% of the normal population. Control lines or animals not representing these extremes represent the greater amount of the population. It seems very likely that in these animals, stress reactivity may be regulated differently than is seen in the extremes of selection procedures. The results of the Con-

trol line birds in this thesis strengthens this opinion. Although the corticosterone differences were clear in the High and Low line birds, the Control line birds actually showed the lowest measured corticosterone levels in both generations and showed the least corticosterone response. In an outbred line of wild type rats the same dependence was observed with male rats that were intermediate in the selection criteria, this sub-group showed the lowest corticosterone levels/response but the highest reactivity in catecholamines (Adriaansen-Tennekes, unpublished results). This higher reactivity from the sympathetic nervous system was related to greater sensitivity for disease development in the MS model, EAE (Chapter 2). In this intermediate group of rats, the suggested mechanisms for the differences in EAE susceptibility were not applicable, as measured by NO production of splenic monocytes, while the results were consistent in the extreme groups (Adriaansen-Tennekes, unpublished results). As this difference in underlying mechanism for EAE susceptibility was only in the intermediate group, and as the second generation of birds from the Control line showed the greatest immunomodulation by diet, the importance of investigating such groups next to the extremes directly becomes evident.

Furthermore, sex of the animals used in experiments that are related to neuroendocrine mechanisms showed sex related differences in regulation as well. Females generally show neuroendocrine regulation that is more alike the reactive males, with high HPA axis reactivity and low SNS reactivity, but females with higher SNS reactivity are seen as well (Panagiotaropoulos et al, 2004; Curtis et al, 2006; Dalla et al, 2008). Again, this difference in neuroendocrine regulation may strongly be related to the role that females play in a population, such as the

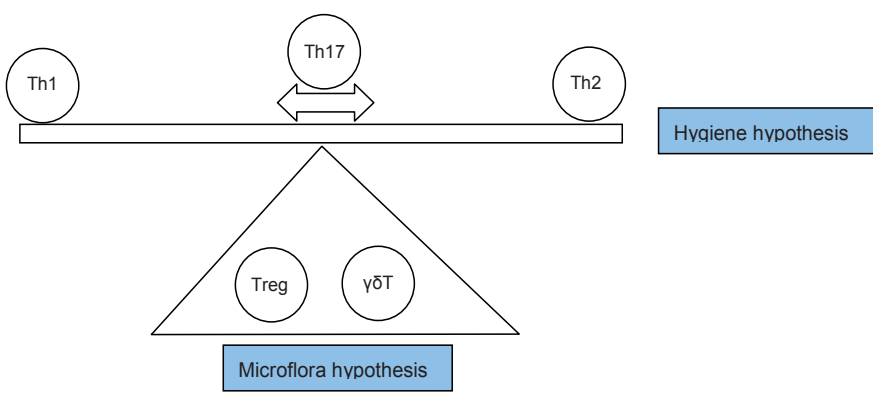

Figure 2. Possible interrelationship between important T cell subtypes with the accompanying hypothesis.

reproduction of offspring requiring an amount of sensitivity for environmental cues (Neumann et al, 2005; Cote et al, 2008; Kokko and Jennions, 2008).

When psychoneuroendocrinology work started, found differences were attributed to the Th1/Th2 dichotomy. As stated earlier, the role of Th17 and Treg's in selection lines, as well as γδT cells, need serious attention to refine this concept with these strong regulatory cells. The interrelationships between these cell types may be as schematically represented in Figure 2.

Although hormone receptor expression has been detected on cells of the immune system, to understand underlying mechanisms that may differ in selection lines hormone receptor expression on sub-types of e.g. T-lymphocytes needs to be investigated. As was seen for the Th2 cell, this sub-type does not express the β2-AR, while the Th1 and B cell do (Nance et al, 2007). When groups of animals differ in their neuroendocrine regulation, as is seen with the selection lines of chicken used in this thesis, differences in hormone receptor expression on sub-types of e.g. T-cells may play an important role in underlying mechanisms of immunomodulation. Therefore examining hormone receptor expression on sub-types of T-cells, such as Treg and Th17, may reveal line-specific mechanisms through which the neuroendocrine system may act on such regulatory cell types.

What do the results in this thesis mean for animal husbandry?

The extrapolation of the results in this thesis to animal husbandry is difficult and complex. Animal husbandry conditions have no resemblance with most natural occurring conditions making it important to take the high population density, the most likely infectious diseases, diet and housing conditions into consideration. Furthermore, animal welfare is becoming a more and more consumer driven topic requiring attention. The use of selection lines differing in behavior, immunity and stress susceptibility may not always coincide with production parameters and therefore with the accompanying economical issues.

As in most European countries, battery cages have been prohibited making husbandry housing comparable to social housing conditions in experiments, but with extreme high population densities. This is an important factor because detrimental behavior in such conditions directly compromises the individual and therefore production. The more docile line of reactive birds would seem to be the best choice under these conditions, but these animals are more stress sensitive. Stress susceptibility could be modified, but would require environmental enrichment and/or more handling by the poultry farmers especially in the starter period, and possibly in the grower period as well. With layer hens, chicks are reared at

other locations than adult layers, making such adaptations in husbandry conditions plausible, but at increasing costs.

The high population density of poultry housing also has consequences for infections. From this perspective, birds with high resistance to infections would be the better choice. In mammals, pro active animals are less prone to bacterial infections but more prone to autoimmune diseases. This would argue for keeping the proactive birds, but instantly the problem occurs with the accompanying more detrimental behavior such as feather pecking. So, which type of animal is most suitable to keep in our husbandry systems?

With the current knowledge as presented in this thesis it would be logical to opt for the reactive animals for several reasons: 1) although several characteristics of these birds, such as lower bacterial resistance and greater stress reactivity, may not result in optimal production capacity, these birds can adapt more readily than proactive birds, because they are more environmentally sensitive; 2) it is desired and possible that the production conditions of the hens laying future layers, can be adapted to enhance the characteristics of the offspring to the production conditions in which the birds will be held, through diet and other mechanisms; 3) reactive birds are more sociable than proactive birds, thus less detrimental behavior at the cost of production; 4) although speculative, it is likely that reactive birds carry more genetic variance than proactive birds, and therefore less genetic information is lost from the population. In the above mentioned reasoning, animals from Control lines or intermediate in the selection procedure are generally not considered due to the lack of insight in such groups and much more focus is on extremes of the population with several risks connected to the analysis of such birds. Such Control line animals, however, may be much more suitable, and thus require further investigation. Overall, further in depth examination of underlying mechanisms and differences in sub-groups of animals will make long term sustainable choices in animal husbandry more reliable.

Interestingly, the results presented in this thesis strongly suggest that much may be gained by triggering, adapting, modulating the mothers of our production animals as our environmental factor, diet altered all the offspring selection lines and not only the more environmentally sensitive Low line birds, as was seen in the first generation. Such an approach will need further investigation prior to implementation on an industrial scale.

While genetic background and/or epigenetic processes on neuroendocrine and immune regulation of the individual form the framework wherein individual immunomodulation by diet can take place, environmental conditions determine if the modulation is beneficial or not.

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English summary

English summary

Individual differences are the basis of diversity within a population increasing survival chances of that population. To classify such differences, the use of concepts such as coping styles are very useful and have been found to adequately represent individuals within natural populations. The use of the coping style concept (as derived from rodent studies), together with a neuroendocrine approach towards immunomodulation by diet of poultry were the basis of this thesis.

Prior work with outbred, wild type rats, revealed that based on characterization of animals in a behavioral paradigm, a classification of individuals could be made coinciding with coping styles. Although not presented in Chapter 2, rats were divided into three groups, the extremes as well as animals showing intermediate behavior in the selection procedure. The results obtained with the *intermediate* group and the group representing *reactive* copers are presented in Chapter 2. The true *proactive* copers, showed intermediate disease susceptibility for the development of EAE, when compared with the intermediate and reactive animals. Interestingly, the role for monocytes in EAE susceptibility was confirmed in the reactive and proactive groups, but not in the intermediate animals.

Previous work with the chicken lines that had been selected for immune responsiveness and were used in the remainder of the experiments presented in this thesis, suggesting possible differences in HPA axis reactivity as was seen within coping styles of rodents. Those intrinsically regulated proactive rats, showed less HPA axis reactivity and more SNS reactivity, while the reactive, more environmentally animals showed the reverse. This difference in neuroendocrine reactivity to stress was examined first to determine if these chicken selection lines used here could represent the same behavioral dichotomy as was seen in rats. Chapter 3 presents the results, wherein the dichotomy in HPA axis reactivity was confirmed. Interestingly, the Control line birds showed the least HPA axis reactivity, coinciding with results found in the intermediate group of wild type outbred rats in Chapter 2.

As animals with different coping styles show differential neuroendocrine regulation, and other studies have shown differential receptor sensitivity to corticosteroids before, line-specific differences of the chicken lines due to stress were examined in Chapter 4. As described by several other groups, the High line birds, with high Ab production and low HPA axis reactivity showed greatest stress effects on cellular parameters of immunity that were strongly correlated with plasma corticosterone levels. On the other hand, the Low line birds with low Ab production and high HPA axis reactivity displayed stress effects on complement

activity. Mild stress may thus affect animals with different coping styles in different manners. The Control line birds were least affected in immune parameters by mild stress, and showed the least rise in corticosterone, implying differential regulation in these animals.

Other groups have proposed that using models with animals representing different stress coping styles may be useful in investigating nutritional immunomodulation. Furthermore, the concept of coping styles wherein the reactive animals are considered to be more environmentally sensitive, suggests that these animals are more sensitive to immunomodulation by diet. In Chapter 5, parameters representing humoral immunity were investigated in two generations of layer hens representing the coping styles as indicated in Chapters 3 and 4, as well as the third intermediate Control line. The hypothesis that the Low line (reactive) birds are most sensitive to immunomodulation by diet was corroborated in specific antibody responses to standard vaccines, as well as a corticosterone response to one of the two used diets. In the offspring generation, the Low line as well as the Control line birds showed altered immunocompetence in a subtype of natural antibodies (NAb) binding to LTA due to the diet offered.

Next to the previously described effects on humoral parameters, cellular immune parameters were measured as well (Chapter 6). Line-specific differences in the three chicken lines were found due to diet as well as generalized effects. In situ monocyte reactivity was enhanced after each diet change, and this effect was greatest in the High line birds. Lymphocyte proliferation was enhanced in one of the two diets in the Low lines birds. Mainly in the Low line birds, several correlations between the cellular parameters and plasma corticosterone levels were found. Interestingly, mainly the controls of the immune parameters measured showed the greatest effects due to diet. The offspring generation was most profoundly affected by the diets as was observed in almost all cellular parameters, with effects in each line, albeit in different magnitudes.

Within neuroendocrine research, often line-specific differences are only found due to triggering of a reaction in the animal. This concept was used to detect possible differences in humoral immunity, that may not be detectable under baseline, stress free conditions. An immunological trigger was used, immunization with KLH, to induce an immune response and parameters of cellular as well as humoral immunity were measured (Chapter 7). Although in the primary immune response of KLH levels of specific antibodies were not altered, almost all other immune parameters showed line-specific differences due to the two diets, with clear differences in kinetics of the immune response. Again, many correlations with corticosterone levels were found. As this analysis was performed in the offspring generation of hens, adaptation of all selection lines was found, but with

line-specific differences.

Within ecological en environmental research much work has been done on maternal effects on offspring. In Chapter 8, coping styles were used as template to explain different adaptation mechanisms found over the two generations of layer hens. As was described previously due to mild stress, the High line birds showed the most accepted adaptation mechanisms that have been found to date, i.e. alteration of sex ratio in offspring. The Low line birds on the other hand, showed more egg production when fed diet A, and the offspring showed an altered HPA axis response to the KLH inoculation. The results strongly suggest that all selection lines showed adaptation to the diets, but that adaptation is done in a line-specific manner.

Overall the results presented in this thesis showed that most effects of stress or diet are found upstream in the immune system, in cellular parameters and/or parameters reflecting innate immunity. Downstream effects (on antibody production) can be found, but a specific trigger is required to make detection possible. In a first generation (enhancing) effects due to diet were mostly found in the Low line birds with a basal level high HPA axis reactivity and a basal low Ab production, confirming the concept of coping styles that such reactive copers are the most environmentally sensitive. On the other hand, all offspring of these hens of the first generation showed clear effects due to diet, strongly suggesting that selection lines with differential HPA axis reactivity adapt to environmental factors in a line-specific manner. While genetic background and/or epigenetic processes on neuroendocrine and immune regulation of the individual form the framework wherein individual immunomodulation by diet can take place, environmental conditions determine if the modulation is beneficial or not.

Nederlandse samenvatting

Nederlandse samenvatting

Individuele verschillen vormen de basis van diversiteit in een populatie, waardoor de overlevingskansen van de populatie worden vergroot. Om de classificatie van individuele verschillen mogelijk te maken, zijn concepten zoals gedrags (coping) stijlen erg bruikbaar en het is herhaaldelijk aantoongetoond dat individuen in natuurlijke populaties deze verschillen reflecteren. Het gebruik van dergelijke concepten, samen met een neuro-endocriene benadering van immuun modulatie door dieet, waren de basis voor dit proefschrift.

Eerder werk met outbred, wild type ratten, heeft laten zien dat een karakterisering van dieren in een gedragstest leidde tot een classificatie van dieren die overeenkomt is met coping stijlen. Hoewel dit niet in Hoofdstuk 2 wordt gepresenteerd, werden ratten in drie groepen ingedeeld, de extremen en de dieren die intermediair scoorden in de gedragstest. De resultaten die zijn verkregen met de *intermediaire* groep en de *reactieve copers* worden gepresenteerd in Hoofdstuk 2. De werkelijke *proactieve* copers lieten intermediaire gevoeligheid voor de ontwikkeling van EAE zien, wanneer ze vergeleken werden met de intermediaire en reactieve dieren. Een interessante bevinding werd gevonden in de rol van monocyten bij de gevoeligheid voor de ontwikkeling van EAE, deze werd bevestigd bij de proactieve en reactieve dieren, maar niet bij de intermediaire dieren.

Uit voorgaand werk met de kippenlijnen die gebruikt zijn voor de rest van de experimenten in dit proefschrift, waren aanwijzingen dat er mogelijke verschillen in HPA as reactiviteit tussen de lijnen zou zijn, overeenkomstig met coping stijlen. Intrinsiek geregelde proactieve dieren, vertonen een lage HPA as reactiviteit en hoge SNS reactiviteit, terwijl de reactieve dieren die meer omgevingsgevoelig zijn het omgekeerde laten zien. Dit verschil in neuroendocriene reactiviteit bij stress is eerst onderzocht om te bepalen of de gebruikte kippenlijnen dezelfde dichotomie in stressgevoeligheid vertegenwoordigen. Hoofdstuk 3 presenteert de resultaten waarin de dichotomie in HPA as reactiviteit werd bevestigd. Een interessante bevinding werd gevonden in de Controle lijn dieren, waarin de laagste HPA as reactiviteit werd gemeten overeenkomend met de intermediaire groep wild type ratten.

Gezien dieren met verschillende coping stijlen verschillende neuroendocriene regulatie vertonen en andere onderzoeksgroepen verschillen in receptor gevoeligheid voor corticosterioden hebben aangetoond, hebben we lijnspecifieke verschillen door stress bekeken in Hoofdstuk 4. Overeenkomstig met andere de resultaten van andere onderzoeksgroepen lieten de Hoge lijn dieren, met een hoge Ab productie en lage HPA as reactiviteit, de grootste effecten van stress zien op

cellulaire immuun parameters die sterk gecorreleerd waren met corticosteron in plasma. Anderzijds lieten de Lage lijn dieren met lage Ab productie en hoge HPA as reactiviteit effecten van stress op complement activiteit zien. Milde stress veroorzaakt bij dieren met verschillende coping stijlen verschillende effecten. De Controle lijn dieren waren het minst beïnvloed in immune parameters door milde stress, en lieten de minste corticosteron toename zien, wat een andere regulatie in deze dieren suggereert.

Verschillende groepen hebben gesuggereerd dat het gebruik van dier modellen waarin coping stijlen worden vertegenwoordigd behulpzaam zouden kunnen zijn bij het onderzoeken van immunomodulatie door dieet. Bij coping stijlen worden de reactieve dieren beschouwd als meer omgevingssensitief, wat zou kunnen impliceren dat de reactieve dieren gevoeliger zouden kunnen zijn voor immunomodulatie door dieet. In Hoofdstuk 5 zijn immuun parameters die humorale immuniteit weergeven onderzocht in twee generaties leg hennen met verschillende coping stijlen, en een derde intermediaire Controle lijn leg hennen. De hypothese dat de Lage lijn, reactieve hennen het meest sensitief zouden zijn voor immunomodulatie door dieet werd gevonden in de specifieke Ab voor de standaard vaccinaties en de corticosteron response van een van de twee onderzochte diëten. Bij de nakomelingen, tweede generatie, lieten zowel de Lage lijn en Controle lijn dieren een verschil in immuun competentie zien bij een subtype Natuurlijke Ab (die LTA bindt) ten gevolge van dieet.

Naast de voorgaand beschreven effecten op humorale parameters, zijn cellulaire parameters ook gemeten (Hoofdstuk 6). Zowel lijnspecifieke effecten door dieet zijn gevonden, en algemene effecten werden gezien. In situ monocyten reactiviteit was toegenomen na elke voerverandering, en dit effect was het grootst in de Hoge lijn dieren. Lymfocyten proliferatie was toegenomen in een van de twee onderzochte diëten bij de Lage lijn dieren. Vooral in de Lage lijn dieren, werden verschillende correlaties gevonden tussen de gemeten corticosteron in plasma en cellulaire parameters. De in vitro controles van de bepaalde immuun parameters lieten de grootste effecten ten gevolge van dieet zien. De nakomelingen, oftewel de tweede generatie, lieten de grootste effecten ten gevolge van dieet zien in bijna alle cellulaire parameters, met effecten in alle lijnen, enkel met verschillende mate.

Binnen neuroendocrien onderzoek worden lijnspecifieke effecten alleen gezien wanneer een reactie van het dier wordt uitgelokt. Dit concept werd gebruikt om eventuele effecten in humorale parameters te meten, die onder normale stress-vrije condities niet meetbaar zijn. Een immuun trigger is gebruikt, KLH, om een immuun respons op te wekken, waarna humorale en cellulaire parameters werden bepaald (Hoofdstuk 7). Hoewel er geen verschil was in de primaire immuun

respons van KLH specifieke Ab, lieten alle andere immuun parameters lijnspecifieke verschillen zien ten gevolge van de twee diëten met duidelijke verschillen in de kinetiek van de immuun response. Ook hier werden meerdere correlaties met plasma corticosteron gevonden. Deze resultaten werden gevonden in de nakomelingen generatie en duiden op adaptatie van alle lijnen, maar met lijnspecifieke verschillen.

Binnen ecologisch en milieu onderzoek wordt veel onderzoek gedaan naar maternale effecten op nakomelingen. In Hoofdstuk 8 zijn coping stijlen gebruikt als kapstok om de verschillende adaptatie mechanismen te verklaren die zijn gevonden in de twee generaties leg hennen. Zoals eerder werd gezien ten gevolge van milde stress, werd in de Hoge lijn dieren de meest geaccepteerde mechanisme gevonden nl. een verandering van de sexe ratio bij de nakomelingen. De Lage lijn dieren gevoerd met dieet A produceerden meer eieren, en de nakomelingen vertoonden een veranderde corticosteron respons op de KLH inoculatie. Deze resultaten suggereren dat alle selectie lijnen adapteren ten gevolge van de diëten, echter de adaptatie gebeurt op een lijnspecifieke manier.

In conclusie, de resultaten gepresenteerd in dit proefschrift laten zien dat de meeste gemeten effecten ten gevolge van stress of dieet worden gevonden upstream in het immuun systeem, nl. in cellulaire en/of parameters die innate immuniteit weergeven. Downstream effecten (bijv. op Ab productie) kunnen gevonden worden, echter een stimulus is nodig om potentiële verschillen meetbaar te maken. In een eerste generatie werden de meeste effecten gevonden in de Lage lijn dieren met hoge HPA as reactiviteit en lage Ab productie, wat een bevestiging is van de het concept van coping stijlen dat dergelijke reactieve dieren meer omgevingssensitief zijn. Daar en tegen waren in alle nakomelingen van de eerste generatie meetbare effecten ten gevolge van de diëten, wat sterk suggereert dat selectie lijnen met verschillende HPA as reactiviteit adapteren op omgevingsfactoren echter op een lijnspecifieke manier. Daarmee bepalen de genetische achtergrond en/of epigenetische processen de neuroendocriene en immunologische kaders waarin bij een individu immuun modulatie door dieet kan plaatsvinden, echter omgevingsfactoren zijn bepalend of de aanpassingen wel of niet beter voor het dier zijn.

Dankwoord

Dankwoord

Zoals in elke promotie zijn ook in deze de nodige “up’s and down’s” geweest: het ontdekken dat ik de ziekte van Ménière heb, mijn ontslag bij mijn vorige werkgever, het schrijven voor een ander, en alles combineren met Emma en een huishouden. Ik kan alleen maar concluderen dat IK een echte stresskip ben. In 1992 ben ik een eerste promotie gestart en hier in Wageningen zijn de eerste en de huidige tot een succesvol eind gekomen. Hoewel het (meeste) werk door de promovendus gedaan wordt, zijn er nog vele manieren waarop anderen meehelpen met de tot stand koming van alle resultaten en het proefschrift. Alleen had ik het nooit gered.

Op deze plek wil ik graag het Ministerie van LNV bedanken voor het nemen van de stap om dit werk inhoudelijk en financieel te ondersteunen. De Rabobank en Triodos bank hebben opgevolgd door medefinanciering. Zo ook wil ik alle projectpartners bedanken voor de prettige samenwerking: bedankt ADP en CBI van het WUR, LBI, Rikilt en TNO. Eveneens wil ik de leden van de Begeleidings-commissie bedanken voor hun kritische blik en met name Henk van Loveren en Raymond Pieters.

Het heeft erom gespannen of het boekje wat nu voor u ligt de huidige vorm en inhoud zou hebben. De inhoud en benadering van dit proefschrift is er één die mij al jaren aan het hart ligt en waar ik heel veel plezier aan beleef om me in deze complexe materie te storten. Het is mede dankzij Martin Scholten dat dit proefschrift tot dit resultaat heeft geleid en daar ben ik Martin erg dankbaar voor, eindelijk afsluiting van twee promoties.

Als echte stresskip die erg omgevingsgevoelig is, zijn vooral de omstandigheden belangrijk geweest om deze promotie tot een goed eind te kunnen brengen. Zonder alle steun en begrip van mijn promotor, Huub Savelkoul, had ik dit nooit afgemaakt. Huub, ik kijk heel erg uit naar alle (onderzoeks-)plannen die we voor de toekomst hebben en ik waardeer de ruimte en mogelijkheden die je biedt aan deze parttimer. Zo ook, heeft mijn co-promotor Henk Parmentier niet alleen inhoudelijk en praktisch erg veel geholpen, maar vooral alle lol die we hebben gehad, maar ook alle begrip heeft deze stresskip erdoorheen geholpen. Henk, welk vervolg onderzoek dan ook, is niet compleet als jij niet mee doet. Heren, ik hoop met jullie nog vaak flinke discussies te kunnen aangaan over de interpretatie van resultaten, want wat hebben we dan een plezier. Iedereen kan er dan van meegenieten……

Ook Bas Kemp, Eddy Decuypere en Jaap Koolhaas hebben een belangrijke rol gespeeld bij de totstandkoming van dit proefschrift, ieder op een heel eigen manier, heren: Bedankt! Jaap, je rol bij mijn promotie maakt de afronding van

twee promoties compleet. Ik hoop dat we in de toekomst de samenwerking kunnen voortzetten.

Ger, Mike, en Adrie zijn mijn steun en toeverlaat geweest om alle bepalingen in te kunnen zetten die er gedaan zijn. Heel simpel, zonder jullie hulp hadden we nooit zoveel kunnen doen! Maar ook daarna, het verwerken van alle monsters en de vele reeksen elisa's, Ger, je hulp was onmisbaar. Marleen heeft mij zo'n beetje alles geleerd over kippen wat er te leren valt en haar toewijding en accuratesse bewonder ik ten zeerste. En Hilda, mijn kamergenoot. Je weet niet half hoe fijn het is dat er nog iemand rondloopt die helemaal herkent hoe ingewikkeld het kan zijn om alles thuis met werken te combineren. Allemaal bedankt!

Het is soms erg lastig om als parttimer bij alles aanwezig te zijn wat van belang kan zijn voor je promotie in welke vorm dan ook, dat is dan ook vaak niet gelukt. Hoewel ik er erg trots op ben dat ik deze promotie in 4 jaar heb afgerond, was het minder soepel geweest wanneer Prescilla niet had geholpen waar ze maar kon. Prescilla, ik dank je daarvoor, en ook omdat mijn boekje echt helemaal naar mijn zin is geworden! Geniet met volle teugen van Sanne, want voordat je het weet is ze groot. Ook Joop heeft een onmisbare bijdrage geleverd aan de vormgeving van dit boekje, als trendzetter van de "hoofdstukblokjes": goed voorbeeld doet volgen!

Voor alle stafleden, promovendi en studenten van ADP en CBI, voor jullie ben ik meer een naam dan een collega geweest. Helaas heeft alle stress, politieke toestanden en mijn gezondheid tijdens deze promotie ertoe geleid dat ik alles behalve gezellig was en daarvoor mijn oprechte excuses. Sinds ik weer wetenschappelijk de ruimte heb gekregen, realiseer ik mij pas hoe erg zelfs. Dankzij de mogelijkheden die er voor mij met mijn beperkingen zijn gecreëerd, is het plezier weer terug en kunnen jullie rekenen op een gezellige collega (ja Gerco, ik kom nu wel mee lunchen)!

Zoals ik in het begin al aangaf, er zijn vele manieren waarop anderen meehelpen bij een promotie. Oma Janny en oma Olga hebben ondanks alle eigen fysieke lasten bij gesprongen wanneer ik maar vroeg. Zo ook hebben onze buren, Claudia en Hendrik-Jan, Corrie en Wim een hele grote en belangrijke bijdrage geleverd. Wetende dat Emma steeds in hun handen was, gaf de rust en ruimte om alles te doen wat er gedaan moest worden en alles wat er in mijn hoofd zat op papier te zetten. Het zijn mensen zoals zij die recht doen aan het gezegde: "een goede buur is beter dan een verre vriend".

Lieve hutseflutsie, Emma dus. Inmiddels ben je alweer 7 en kun je zelf lezen wat hier staat voor jou en over jou. Hoewel jij het niet altijd leuk vond dat ik zo bezig was, heb je toch heel erg lief meegewerkt en je best gedaan om mij te laten werken. Ook jij hebt dus geholpen in het maken van dit boekje. Dank je wel

broodje aap!

En dan “last but not least”: Rob. Schat, hoewel ik steeds riep: “als het promoveren, in welke vorm dan ook, ten koste van teveel gaat, dan stop ik ermee”, riep jij terug: “je moet het nu afmaken”. Je morele steun, begrip wat het voor mij betekent en hulp thuis om dit mogelijk te maken, zal ik je eeuwig dankbaar voor zijn, want wat ben ik trots op wat hier ligt!

I DID IT!!

Personalia

Curriculum vitae
List of publications
Education certificate

Curriculum vitae

Ruth Adriaansen-Tennekes was born in Delft on April 8th, 1965. Three months after she was born, she moved to State College, Pennsylvania in the United States, where she lived for 12 years with her familiy. In 1986, after graduating from Atheneum F. de Munnick in Utrecht, she started her study in Biology at the Rijks Universiteit Groningen.

After the completion of her study (1992), the author started to work on a PhD project on individual differences in behavior in relation to differences in immunity which was a NWO project and collaboration between the University of Utrecht and the Rijks University of Groningen. In the last year of her PhD, before starting the writing of her thesis she switched careers and started working in the pharmaceutical industry as Clinical Research Associate, later as Project Manager and lastly as Outsourcing Manager. After several years in the "Industry" where she met her husband, the author returned to her great passion, fundamental research (2001). Through the project presented in this thesis, a collaboration with Prof. Savelkoul was initiated.

Currently, the author is employed as a scientist at the department of Cell Biology and Immunology, headed by Prof. Dr. Huub Savelkoul.

List of Publications

Kavelaars, A, C.J. Heijnen, R. Tennekes, J.E. Bruggink, and J.M. Koolhaas. 1999. Individual behavioural characteristics of wild-type rats predict susceptibility to experimental autoimmune encephalomyelitis. Brain Behavior and Immuninity 13(4):279-86.

Baars, E.W., R. Adriaansen-Tennekes, and K. Eikmans. 2005. Safety of homeopathic injectables for subcutaneous administration as used in Homeopathic and Anthroposophic Medicine. Journal of Alternative and Complementary Medicine 11(4):609-161.

J. Bloksma, J., R. Adriaansen-Tennekes, M. Huber, L.P.L. van de Vijver, T. Baars and J. de Wit. 2008. Comparison of Organic and Conventional Raw Milk Quality in The Netherlands. Biological Agriculture and Horticulture 26: 69–83.

Adriaansen-Tennekes, R., E. Decuypere, H.K. Parmentier, and H.F.J. Savelkoul. 2009a. Chicken lines Selected for their Primary Antibody response to SRBC show differential HPA axis responsiveness to mild stressors. Poultry Science 88: 1879-1882.

Adriaansen-Tennekes, R., G. de Vries Reilingh, M.G.B. Nieuwland, H.K. Parementier, and H.F.J. Savelkoul. 2009b. Chicken lines divergently selected for Antibody responses to SRBC show line-specific differences in sensitivity to immunomodulation by diet, part I: humoral parameters. Poultry Science 88: 1869-1878.

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Management Skills Training (1.2 ECTS)	
Membership of committee 'Organic, More Healthy?'	2005-2007
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The cover of this thesis is a photoshoped photograph of the authors own chickens. Within this flock the relationship between feather colour and behaviour is apparent, as well as a difference in maternal care. Observing my own flock was helpful in approaching the found results in this thesis in different ways.

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